Seradate:

The development and validation of an oral fluid collection device

Thesis submitted in accordance with the requirements of University of Liverpool for the degree of Doctor in Philosophy

by

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Acknowledgments

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Abstract

Recent interest has grown in regard to non-invasive testing and oral fluid diagnostics. The use of oral fluid allows for a mobile diagnosis service that is not restricted to an assigned place allowing for testing to be conducted from 'bedside to roadside'. The development of our unique collector Seradate, an oral fluid collection device prototype that was designed with the intention of collecting oral fluid that is rich in oral mucosal transudate (OMT). OMT is an unique fluid that is presented from the gingival crevice and contains serum-based compounds. Due to low levels of OMT presence within oral fluid our ideas in design were formed on postulated and experimental models of inducing the release of OMT. The Seradate was tested alongside current marketed oral swabs for its effectiveness in monitoring levels of OMT-derived immunoglobulins present within oral fluid. Further validation work was conducted on the monitoring of stress hormones and the determination of smoking status. Our findings showed that the Seradate was an adequate alternative to current marketed oral swabs in the monitoring of oral based constituents and with a few minor modifications the Seradate can be used as an alternative to current oral swabbing methods.

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Abbreviations

APS	Ammonium persulfate
BSA	Bovine serum albumin
CF	Crevicular fluid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GCF	Gingival Crevicular Fluid
HCL	Hydrochloric Acid
H_2SO_4	Sulphuriv Acid
HRP	Horse radish peroxidise
IAA	Iodoacetic acid
IEF	Iso-electro focussing
IgA	IgA Immunoglobulin A
IgG	IgG Immunoglobulin G
IgM	IgM Immunoglobulin M
OFCD	Oral Fluid Collection Device
OMT	Oral Mucosal Transudate
PBS	Phospahte buffered saline
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIgA	Salivary immunoglobulin A

TMB	Tetra methyl benzidine

1 INTRODUCTION

Medical diagnostics have been used to monitor disease, infection immunisation status, hormonal imbalances and drugs using various fluid matrices found within the human body. The collection of fluid matrices for diagnosis has been simplified into 2 categories for the purposes of this thesis invasive collection and non-invasive collection. Invasive procedures are physical interventions that require breaking of the skin. These procedures are used in the collection of blood, cerebral spinal fluid and organ biopsies. The procedures can be restrictive in terms of compliance issues, and procedures require trained individuals and collection requires a clinical setting. Non-invasive procedures may require partial intervention by a third party but involve no physical breaking of the skin. These procedures include the collection of urine and oral swabbing. Non-invasive procedures are mainly self-directed and can be done in a more relaxed surrounding with minimal supervision.

Recent interest has grown in regard to non-invasive testing and oral fluid based diagnostics. The use of oral fluid allows for a mobile diagnosis service that is not restricted to an assigned place allowing for testing to be conducted from "Bedside to Roadside" (Malamud 1997; Humphrey and Williamson 2001; Vyse, Cohen et al. 2001; Cameron and Carman 2005; Chiappin, Antonelli et al. 2007). With recent advancements in computational and analytical sciences, oral fluid samples of low sample volume and protein concentration can be screened for systemic disease, illicit and therapeutic drugs and hormones. Oral fluid testing provides ease of access, ease of use, patient confidentiality and acceptability (Cordeiro, Turpin et al. 1993; Malamud 1997; Uitto 2003; Cameron and Carman 2005). Patient acceptability is with reference to

compliance issues where intravenous collections are problematic such as in the elderly, young children, haemophiliacs and venous-compromised individuals.

The acceptance of oral fluid as an alternative matrix to blood has advanced over the past 20 years. This has been linked to the profiling of the oral fluid proteome which is nearing completion, and has allowed the discovery of serum based markers within the matrix that are presented by oral mucosal transudate (OMT) also known as gingival crevicular fluid (GCF). Oral Fluid and its constituents have provided a variety of disease markers that are of local (Intra-oral) and systemic origin (Streckfus and Bigler 2002; Van Nieuw Amerongen, Bolscher et al. 2004; Aurer, Jorgic-Srdjak et al. 2005).

1.1 Origins of Oral Fluid and OMT

Oral Fluid

Oral fluid (whole saliva) is secreted from multiple salivary glands within the oral cavity (Figure 1.1).This includes major salivary gland secretions from the parotid gland, submandibular gland, sublingual gland and minor salivary glands that lie beneath the oral mucosa (Humphrey and Williamson 2001; Schipper, Loof et al. 2007).

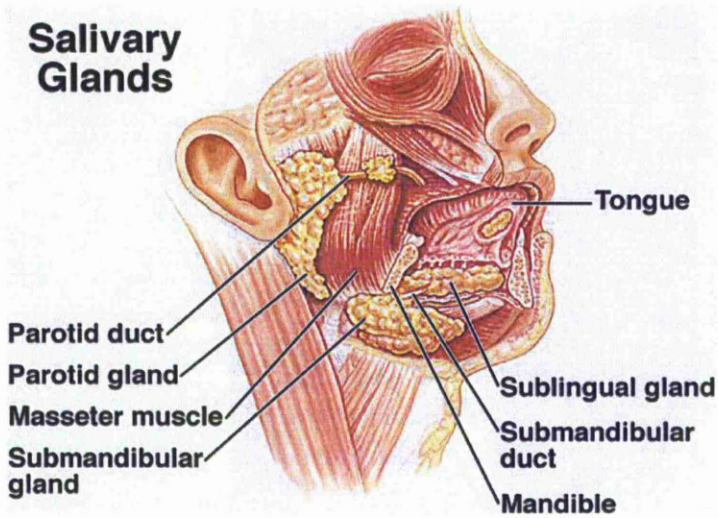

Figure 1.1 The anatomical positioning of the major salivary glands. The parotid gland, the submandibular and sublingual gland. This image was modified from McGraw Hill Companies.

The minor salivary glands lie beneath the oral mucosa these include the serous von Ebner glands located within the connective tissue under the circumvalate papillae and the Blandin-Nühm mucous glands (Humphrey and Williamson 2001).

The consistency of oral fluid can either be serous or mucous, this is dependent on the involvement of specific salivary glands.((This is effectively a ratio of gland involvement that is accounted for by 65%, 23%, 8% to 4% for the submandibular gland, parotid gland, Von Ebner gland and sublingual gland secretions in that order (Chicharro JL 1998; Hu, Denny et al. 2004; Chiappin, Antonelli et al. 2007)).

Secretions from both the submandibular and sublingual salivary glands enter the oral cavity through Wharton's duct. The collection and evaluation of the secretions from the individual salivary glands are useful for the detection of gland-specific pathology caused either by infection or obstruction (Mandel 1993; Aps and Martens 2005). The salivary glands are formed of two specific specialised epithelial cell regions: the acinar and ductal regions. The acinar region is a hub for protein synthesis and protein secretion. Amino acids enter the acinar cells by active transport, and after intracellular protein synthesis, the newly formed proteins are stored in storage granules until they are released in response to secretory stimulation (Kaufman and Lamster 2002; Aps and Martens 2005). Acinar fluid secretion can occur by active transport of anions into the salivary lumen and the movement of water is propagated by an osmotic gradient from interstitial fluid into salivary lumen. The acinar fluid is isotonic and formed within local vasculature. Acinar cells are permeable to water whereas ductal cells are not.

Ductal cells absorb most of the Na^+ and Cl^- ions from primary salivary secretion and secrete small amounts of K^+ and HCO_3^- and some proteins. This modifies the final salivary secretion to be Hypotonic (Baum, 1993). Salivary secretion is maintained by the autonomic nervous system (parasympathetic and sympathetic).

The signalling mechanism involves the binding of neurotransmitters acetylcholine and nor-epinephrine to plasma membrane receptors and signal transduction *via* guanine nucleotide-binding regulatory proteins (G-proteins) and activation of intracellular calcium signalling mechanisms. There are several routes by which serum constituents that are not part of the normal salivary composition (*i.e.* drugs and hormones) can reach saliva (Figure 1.2). Within the salivary glands, transfer mechanisms are by intracellular routes (passive diffusion) and extracellular routes (ultra-filtration). A serum molecule reaching saliva by diffusion must cross five barriers: the capillary wall, interstitial space, basal cell membrane of the acinus cell or duct cell, cytoplasm of the acinus or duct cell, and the luminal cell membrane (Aps and Martens 2005).

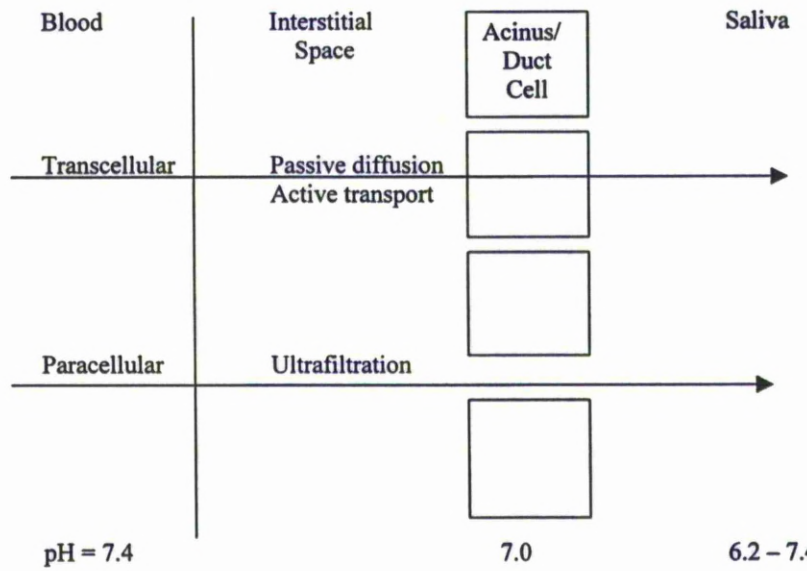

Figure 1.2: The transport of molecules that not part of the normal salivary secretion from serum to saliva is transcellular route (passive diffusion and active transport) and paracellular route (ultrafiltration) through tight junctions.
(Figure adapted from Haeckel and Hanecke 1996)

Oral Mucosal Transudate

Oral mucosal transudate (OMT) is also known as gingival crevicular fluid (GCF), crevicular fluid (CF) and gingival fluid (GF) (Cordeiro, Turpin et al. 1993; Malamud 1997; Uitto 2003; Cameron and Carman 2005) (Figure 1.3). It has been termed as an inflammatory exudate or interstitial transudate. Both forms exhibit slight differences not only in the presence of serum based proteins but also elevated levels of albumin and inflammatory cytokines. These are linked to localised disease of the gingivae that can range from gingivitis to periodontitis (Griffiths 2003).

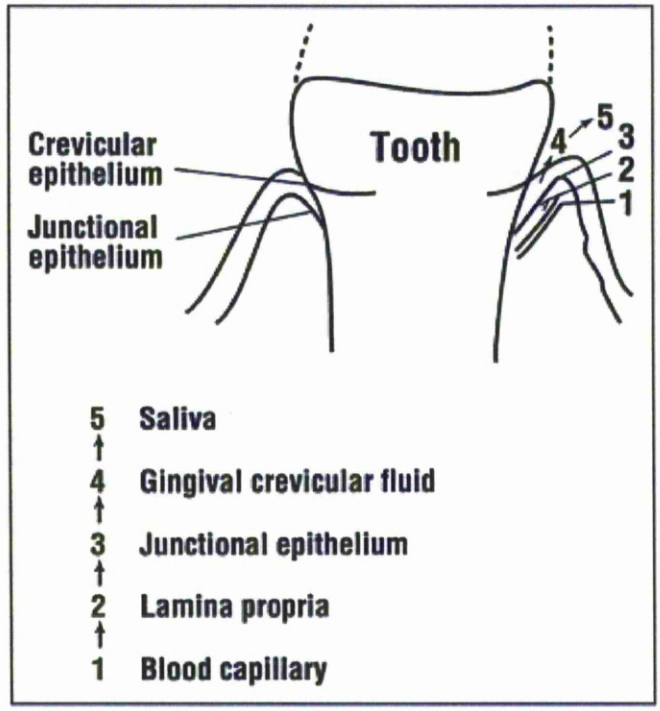

Figure 1.3: The flow of gingival fluid by which components of serum can reach the dental and epithelial surfaces of the mouth.
(Adapted from Malamud 1997)

OMT production and release was postulated by Brill and Krasse, stating that OMT is an important component of the protective mechanisms of the crevicular region of the gingival sulcus (Brill N 1958). The theory suggested that stimulation of the gingival margin was important for the maintenance of gingival health. To test this hypothesis of changes in capillary permeability healthy gingivae were induced either mechanically using abrasion or chemically with histamine. The experiments showed that stimulation of the gum surfaces caused an increase in the flow of OMT and an increase in vascular permeability. This coincides with natural formation of OMT within a clinically healthy gingival crevice. Under natural oral conditions the presence of bacterial plaque would result in the accumulation of high molecular weight molecules creating an osmotic gradient that would induce the release of interstitial fluid from the connective tissue to the gingival sulcus. This finding was supported by the application of phosphate-buffered saline (PBS) containing homologous serum albumin on the gum surface causing a 100% increase in the volume of OMT produced. In contrast, the application of PBS alone caused no such effect (Stoller, Karras et al. 1990).
The theory of OMT being an interstitial transudate was suggested by Alfano from the hypothesis postulated by Pashley (Alfano 1974; Pashley 1976). The theory suggested that OMT production is governed by the passage of fluid from capillaries into the tissues as a capillary filtrate and the removal of this fluid by the lymphatic system (lymphatic uptake). When the rate of capillary filtrate exceeds that of lymphatic uptake, fluid will accumulate as oedema and/or leave the area as OMT. It was also postulated that the initial fluid produced could simply represent interstitial fluid which appears in the crevice as a result of an osmotic gradient. The initial pre-inflammatory fluid was considered to be a transudate,

and on stimulation, this changed to become an inflammatory exudate (Tollefsen and Saltvedt 1980; Griffiths 2003).
The levels of OMT protein concentration shown in studies states that inflamed or diseased gingivae protein levels are comparable to the protein concentration of serum. Further analysis showed that proteins were significantly lower in comparison to serum, but with a strong co-variation between the proteins studied in the two fluids, suggesting that OMT represents an inflammatory exudate of serum. Due to the relatively small volume of OMT produced, especially in healthy gingivae the protein concentration was similar to that of interstitial fluid. This would be consistent with the hypothesis by Alfano that the initial fluid accumulation represents a transudate of interstitial fluid produced by an osmotic gradient and that the later fluid represents a true exudate (Alfano 1974; Griffiths 2003). The presence of serum constituents in oral fluid are largely attributed to OMT outflow. The levels of serum derived proteins are associated with gingival health. Oral disease such as gingivitis and periodontitis would cause greater inflammation of the gum surfaces resulting in OMT that is analogous with serum and not interstitial fluid (Griffiths 2003). These findings are seen in chronically inflamed gingival tissue as healthy gingivae only occasionally responded to these stimuli thus suggesting an alternate pathological origin of OMT.

1.2 The functions of oral fluid and the importance of OMT

Our main components of interest in oral fluid are saliva and oral mucosal transudate.(Humphrey and Williamson 2001; Chiappin, Antonelli et al. 2007). The use of oral fluid as a method of diagnosis shows a lower risk of being altered by the patient in comparison to urine.

The primary function of oral fluid is a lubricant, so as to keep the oral cavity hydrated this helps in the initial stages of digestion, the presence of alpha-amylase helps with the initial stages of digestion. The protective properties of oral fluid within the oral cavity include numerous antimicrobial constituents such as statherins that are protease inhibitors and involved in the maintenance of tooth enamel mineralisation, mucins are low molecular weight proteins that bind to bacteria and viruses to ensure oral cavity clearance, the lack of mucins would result in oral fluid to lose its resistance and the oral cavity will be more susceptible to inflammatory and infectious complications. IgA provides further anti-bacterial protection by agglutination to bacteria. Its origins are centred in Peyer's patches, lymphatic cells of mucous and tonsils (known collectively as mucosal associated lymphoid tissue MALT) and lactoferrin binds to iron ions that are vital for bacterial survival. There are additional proteins such as histatins and polypeptides all of which exhibit anti- bacterial and anti-fungal properties.

Other components present in Oral Fluid include expectorated bronchial and nasal secretions, serum and blood derivatives from open wounds, viruses, fungi from oral flora, desquamated epithelial cells and food debris(Alfano 1974; Messana, Cabras et al. 2004; Messana, Inzitari et al. 2008). OMT has an important function in the maintenance of antimicrobial defence of the

periodontium and the structural integrity of the junctional epithelium. It also acts as mediator in the transport of bacterial products within the periodontal environment and the outward movement of host derived products. OMT provides a measure of host mediated response to microbial plaque and its constituents can be analysed to measure transitions from healthy to disease status of the gingivae .The ability of OMT to present systemic serum derivatives is important in measuring links between intra/inter oral health and systemic well being (Carneiro, Venuleo et al. 2012).

1.3 Collection of oral fluid

The role of oral fluid and its constituents are being studied due to its variability between and within individuals. The collection procedures dictate the quantity of oral fluid collected; this process can be linked to Pavlov's theory of conditional reflexes. The link between innate or conditioned reflex will have a direct influence on the volume of oral fluid released but will have no effect on overall consistency of fluid collected (Pavlov 1927/1960).
Unlike blood or urine oral fluid can be separated by its individual secretions.
Parotid gland secretions are collected with the Carlson-Crittenden collector that is placed at the opening of the Stenson duct and held in place with suction. Sublingual and submandibular secretions are collected using suction or by an aggregator. The secretions of individual salivary glands show an increase and decrease of particular proteins in relation to exogenous factors such as stress, exercise and diurnal effects.
Our focus lies in the collection of oral fluid and OMT; oral fluid can be collected as a stimulated sample, unstimulated sample or by an oral fluid collection device (OFCD).

Unstimulated oral fluid collection

Unstimulated oral fluid can be obtained either by the drooling method. This is to allow oral fluid to accumulate on the floor of the mouth and to drip off the lower lip into a collection tube (Martin and Burgen 1962; Mandel 1993; Aps and Martens 2005), or the spiting method (expectoration), in which the subject expectorates oral fluid into a tube.(Martin and Burgen 1962; Aps and Martens

2005). Unstimulated oral fluid flow rate is affected by levels of hydration, but also by olfactory stimulation, exposure to light, body positioning, and seasonal and diurnal factors.

Stimulated oral fluid collection

The collection of stimulated oral fluid involves moving the tongue around the oral cavity, pursing of the lips and rubbing of the cheeks. The method of stimulation can be enhanced by chewing on paraffin wax, rubber bands or chewing gum and the placement of an absorbent pad are termed as mechanical stimulation methods(Granger,Schwartz et al. 1999; Dawes, Tsang et al. 2001; Meurman, Rantonen et al. 2002). Gustatory stimulation of oral fluid can also be used by the addition of lemon drops or citric acid on the tip of the tongue, the fluid is collected by expectoration, drooling, suctioning or swabbing (Granger, Schwartz et al. 1999).

Oral fluid collection devices

Oral fluid collection devices (OFCDs) were developed to standardise and regulate oral fluid collected for its use in biomedical research and field-based screening. (Malamud 1997; Cameron and Carman 2005). There are a variety of OFCDs currently marketed specifically for the collection of oral fluid. The collection technique largely uses a swabbing method directed to gum surfaces and inner cheek. To achieve a standardised volume of oral fluid the collectors use absorbent food safe materials such as cotton and specially treated hydrophilic polymers (sintered plastics, fibrous polymers) to adequately collect and release oral fluid. Once the sample has been collected the collector is placed into a tube or vial to avoid user contamination. An antibacterial/ antifungal agent can be used for sample stability to prevent the actions of bacterial and salivary proteases. The sample is then processed for testing purposes. There are countless commercial OFCDs, for the purposes of the thesis we will focus on Orasure OFCD, Salivette OFCD, Oracol OFCD and Concateno Certus OFCD. These devices have been advocated for oral fluid collection and the testing for therapeutic drugs, drugs of abuse, hormones and vaccine and disease antibodies(Alfano 1974; Malamud 1997; Vyse, Cohen et al. 2001; Kidd, Midgley et al. 2009; Kozaki, Hashiguchi et al. 2009; Almela, Hidalgo et al. 2011).

Orasure oral fluid collection device

The Orasure oral fluid collection device is an FDA approved OFCD (Figure 1.4).

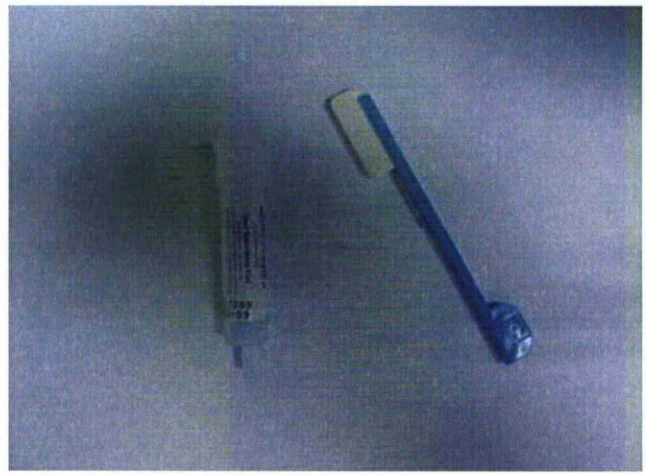

Figure 1.4 Orasure oral fluid collection device

The collector is composed of an absorbent cotton pad affixed to nylon stick. The pad is treated with a buffered salt solution (pH 7.2) comprising of 0.1% gelatine, 3.5% sodium chloride, 0.3% citric acid, 0.1% potassium sorbate and 0.1% sodium benzoate). The pad is positioned between the lower cheek and gums, the salts contained within the pad create a hypertonic environment allowing transfer of buccal and gingival tissue interstitial fluid to migrate across the mucosal surfaces and be collected on the pad. The use of gelatine within the pad is to render irreversible binding of antibodies to the cotton surface. The pad is kept in place for a period of 2-5 minutes to allow for complete saturation and placed within a tube containing a preservative solution (0.5% Tween-20, 0.01% chlorohexidine digluconate) (Cordeiro,Turpin et al.1993; Malamud 1997;Cameron and Carman 2005). Various studies were

conducted utilising this collector and have aided greatly in the assessment of disease states such HIV, Hepatitis B/C and drugs of abuse (Cameron and Carman 2005; Langel,Engblom et al. 2008; Chang, Cohen et al. 2009).

Salivette

The Salivette was developed by Sarstedt AG & Co, it is an absorbent cylindrical collector composed of cotton, polyester or polyethylene (figure 1.5). Oral Fluid is collected by placing the cylindrical collector under the tongue or against the cheek. Upon saturation the collector is placed into a tube for transport prior to testing. It uses have been associated with the monitoring of serum free cortisol present within oral fluid, unbound cortisol reflects actual cortisol levels within the body and is a better indicator than its serum counterpart (Krueger, Breunig et al. 1996; Strazdins, Meyerkort et al. 2005; Gröschl and Rauh 2006).

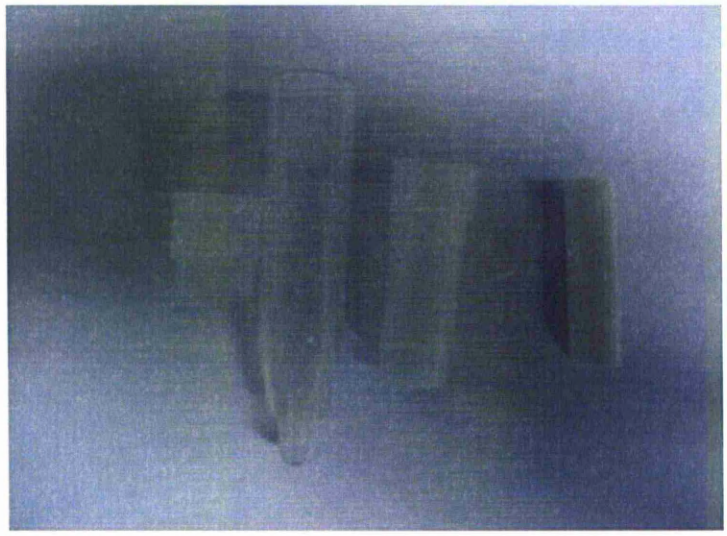

Figure 1.5 Salivette collection device

Oracol

The Oracol saliva collection device was developed by Malvern Medical Developments Ltd (figure 1.6). The Oracol test kit has been designed to collect OF from patients. The kit consists of an absorbent foam swab, its uses have been linked with the monitoring antibodies in the detection of HIV, hepatitis A and B, mumps and rubella and the assessment of cortisol levels in response to stress.(Vyse, Cohen et al. 2001; Morris, Cohen et al. 2002; Langel, Engblom et al. 2008). The sample is collected either by centrifugation or compression of the foam pad.

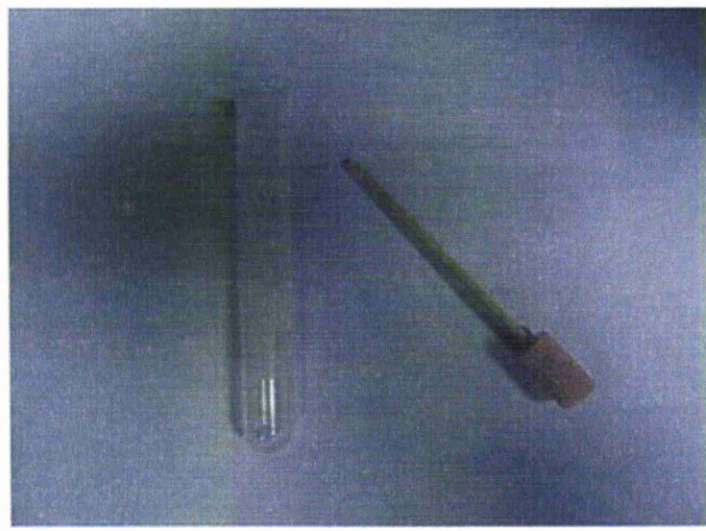

Figure 1.6 Oracol saliva collection device

Concateno Certus

The Concateno Certus collection device is composed of an absorbent material that is attached to a nylon handle (figure 1.7). The device is used in the same manner as the Orasure OFCD. The Certus has a distinct difference in comparison to the other listed collectors as it has a volume adequacy indicator that is incorporated into its handle, that changes from white to blue once sufficient oral fluid is collected (approximately 1ml). The collector is then placed into a vial containing preservative. The Certus OFCD is unique in its processing as it doesn't use centrifugation as its method of extracting collected oral fluid, as the constituents within the preservative help to leach out oral fluid compounds. The uses of the Concateno Certus have not been listed in the literature.

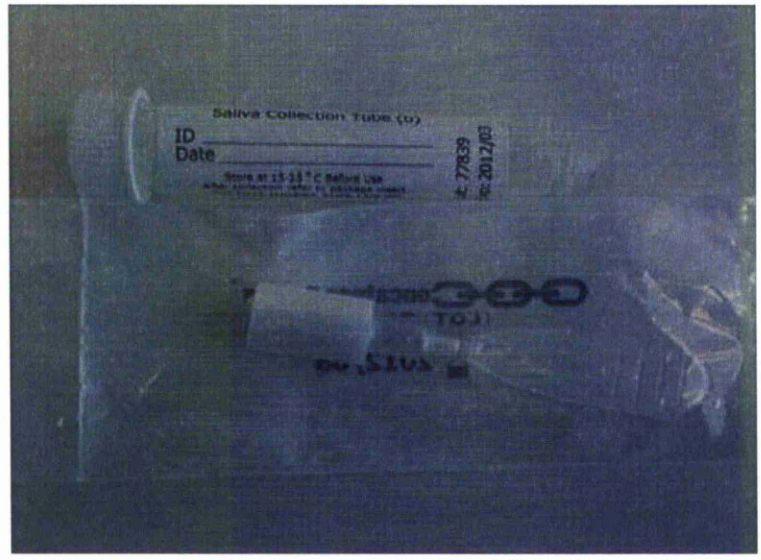

Figure 1.7 Concateno Certus collection device

1.4 Collection of OMT

OMT collection is a non-invasive procedure and requires dental expertise to prevent any trauma to the patient. There are several collection techniques that have been employed for the collection of OMT each technique has its distinct advantages and disadvantages. The techniques can be divided into three methods.

Gingival washing method

The gingival crevice is perfused with an isotonic solution (Hanks' balanced salt solution) of fixed volume. The fluid collected then represents a dilution of crevicular fluid and contains both cells and soluble constituents such as plasma proteins. The technique can be applied to an individual site or group of sites that can divided by into 2 groups of healthy and unhealthy gingivae. The sample is collected by aspiration. As complete sample collection cannot be guaranteed an accurate quantification of OMT volume or composition is not possible as a dilution factor of the final collected sample cannot be determined (Oppenhei.Fg 1970; Skapski and Lehner 1976; Griffiths 2003)

Capillary tubing and micropipettes

The collection site is dried with cotton rolls, capillary tubes of known internal diameter are inserted into the entrance of the gingival crevice. OMT from the crevice migrates into the tube by capillary action and because the internal diameter is known the volume of fluid collected can be accurately determined by measuring the distance which the OMT has migrated. This technique appears to be ideal as it provides an undiluted sample of 'native' OMT whose volume can be accurately assessed. However, it is difficult to collect an adequate volume of OMT in a short period, unless the sites are inflamed and contain large volumes of OMT. To collect a reasonable volume of fluid may, in some instances, mean that collection times from an individual site may exceed 30 minutes and, even then, adequate samples from healthy crevices may be impossible to obtain. It is difficult to conceive that holding a capillary tube at the entrance to a gingival crevice for such lengthy periods ensures an atraumatic collection. A further complication of this technique is the difficulty of removing the complete sample from the tubing. This requires a jet of air, passing a larger fixed volume of a diluting solution through the capillary tube or by centrifugation of the tube (Sueda, Bang et al. 1969; Griffiths 2003).

Absorbent filter paper strips

This is the most common form of collecting OMT the advantages of the technique are that it is quick and easy to use, and can be applied to individual sites of interest. The methods of collection are divided into two forms the intra-crevicular and extra-crevicular technique.

The former involves insertion of the filter strip into the gingival crevice, whereas in the latter the filter strip is laid atop of the gingival crevice region. The intra-crevicular method is the method used most frequently and can be further subdivided depending upon whether the strip is inserted just at the entrance of the crevice or periodontal pocket (superficial) or whether the strip is inserted to the base of the pocket or 'until minimum resistance is felt' (Deep) (figure 1.8).

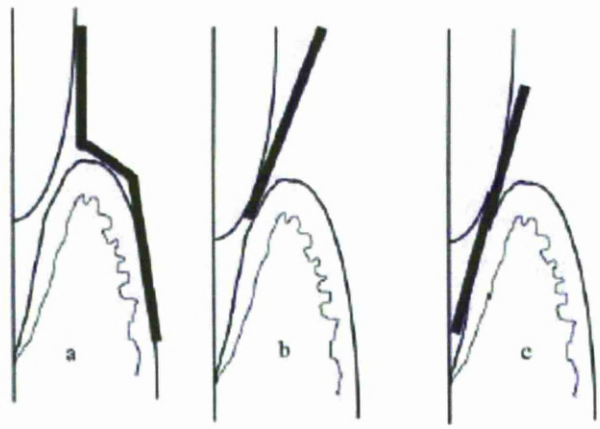

Figure 1.8: An illustration of the positioning of paper for the filter paper strip method of collection (a) Extra crevicular (b) Intra crevicular (Superficial) (c) Intra crevicular (Deep). Modified from Griffiths GS 2000.

In shallow pockets or healthy crevices both intra and extra-crevicular method would represent the same volume. The amount of OMT collected on a strip can be assessed by the distance the fluid had migrated up the strip. This is often taken as a simple linear measurement, but a more accurate value is achieved by weighing the filter paper before and after collection. (Loe and Holmpede.P 1965; Griffiths 2003)

1.5 The profiling of oral fluid proteins

Oral fluid contains a vast array of proteins that are linked to the homeostatic maintenance of the oral cavity. Most proteins and peptides present within oral fluid undergo a complex series of molecular processes, which ultimately define their structures. The onset of these processes occurs at the biosynthetic level within the gland, while terminal processing of the protein/peptides takes place after secretion into the oral cavity. In the lifetime of a salivary protein, four principal phases can be distinguished(Helmerhorst and Oppenheim 2007).

- Phase 1 consists of the basic cellular process of protein biosynthesis, based on its genetic blueprint.
- Phase 2 is characterized by intracellular post-translational modifications prior to secretion of the protein into the ductal system.
- Phase 3 consists of modifications incurred during secretion and transit through the ductal tree.
- Phase 4 represents extensive modifications to salivary proteins after their release into the non-sterile environment of the oral cavity. This last phase has profound consequences for the proteome of oral fluid, since the population of proteins and peptides undergoes continued modifications in the time span between entry into and clearance from the oral cavity.

The definition of the oral fluid proteome is therefore highly variable, dependent on time and the nature and amounts of agents capable of protein/peptide modifications.
The collection of oral fluid and the monitoring of its proteins have been of great interest to scientists on mapping the oral fluid proteome (oralome). Various studies have been conducted to evaluate proteins present within oral fluid. Oral fluid composition is subjective to change intra and inter individually, studies have been conducted to map protein secretions from individual salivary glands and OMT .Oral Fluid proteins are susceptible to degradation upon release from the oral cavity, to improve stability they are treated with protease inhibitors until they can be processed. The maintenance of limiting degradation of collected samples in profiling studies is to limit the presentation of degraded that would not be present within oral fluid in vivo (Amado, Vitorino et al. 2005; Messana, Inzitari et al. 2008).
The profiles of oral fluid proteins have been analysed using 2D gel electrophoresis for qualitative measurements and quantitation has been conducted using LC/GC mass spectrometry. 2D gel electrophoresis is used to screen oral fluid proteins and profiles, its limitations lie in presentation of high molecular weight proteins such as alpha-amylase and albumin which can lead to smearing and distortion of the final profiles and cause inter-variability between gel profiles. To eliminate this problem several methods have directed to the use of effectively removing these proteins from the samples (Amado, Vitorino et al. 2005; Krief, Deutsch et al. 2011).High molecular weight proteins that are transferred in oral fluid that are usually of OMT origin are the systemic immunoglobulins and albumin (Hu, Loo et al. 2007).The protein content of OMT is signified by the level of insult to the gum tissues, within normal gingivae they account for a presence of

30% of serum based proteins as opposed to a presence of 70 % found in periodontic patients. The analysis of GCF proteins and markers for disease is usually hindered by the presence of large molecular weight proteins such as albumin and immunoglobulins, which are presented in oral fluid by OMT Outflow (Carneiro, Venuleo et al. 2012).

1.6 Oral fluid and assessment of disease

Oral fluid and systemic disease

Autoimmune disease is defined by a failure in the autoimmune system that can lead to organ damage. The difficulty in diagnosis lies in the uncertainty of its origin. Sjorgren's syndrome is a common chronic, autoimmune disorder characterized by salivary and lachrymal gland dysfunction, serologic abnormalities, and multiple organ-system changes. Viruses such as HTLV-1 play a role in the pathogenesis of disease Sjorgren's syndrome can be divided into primary and secondary forms. The primary form only affects lachrymal and salivary tissue and the secondary is linked to SLE, rheumatoid arthritis and vasculitis. The effects of Sjorgren's show hypo salivation, causing a decrease in levels of saliva. The effects of hyposalia are extenuated on the tongue, mucous membranes, causing atrophy to papillae, resulting in drying and reddening of the oral mucosa. The biomarkers that are presented in blood and oral fluid such as IL-6, TNF-alpha, and a range of antibodies have been studied, but this has been largely unsuccessful due to a lack of specificity to the presentation of the disease. Recent advances in protein chip technology showed raised levels of lactoferrin, cystatin C and a decrease in alpha-amylase and carbonic anhydrase in Sjorgren's sufferers. Further monitoring of OMT and salivary markers would potentially provide a diagnostic guideline for maintenance of this disorder (Kalk, Vissink et al. 2002; Chiappin, Antonelli et al. 2007).

Cardiovascular disease is a major cause of death world-wide. Markers that are present in oral fluid can be a useful predictor in the monitoring of cardiac health. This would be useful in

postoperative follow-up among patients undergoing cardiovascular surgery. Salivary alpha amylase is an ideal marker as studies have shown that there is a direct link between raised levels of alpha-amylase and heart rate upon the induction of stress. There is a positive relationship between sympathetic nervous activity and corresponding measurements in alpha-amylase (Putignano, Toja et al. 2003)

Oral fluid and viral diseases

In terms of HIV, production of antibodies directed toward specific viral protein epitopes, and the development of technologies capable of measuring these proteins, have facilitated the use of testing for HIV infection in oral fluid. Measuring antibodies directed to HIV that are present in oral fluid by ELISA and confirmatory western blotting, has shown serum sensitivity and specificity. Oral fluid can be used to measure β-2- microglobulin and/or soluble tumor necrosis factor α-receptor levels, and thus assess the disease activity in patients with HIV infection or other chronic inflammatory disease states.
In addition to the above determinations, Oral fluid has also been used for the measurement of other blood borne viruses including Hepatitis C and Hepatitis B (Bull, Kimmance et al. 1989; Malamud 1997; Vyse, Cohen et al. 2001; Cameron and Carman 2005; Landrum, Wilson et al. 2005).

Oral fluid and oncology

With the anatomical proximity of saliva to both pre-malignant and malignant oral neoplasms, oral fluid provides a unique window for the screening of these lesions. As a great number of cancers are related to inactivity of the p53 suppressor gene, this leads to many malignant forms of cancer to develop.

The study of CA-125, a glycoprotein complex that is an often-used marker for ovarian cancer was compared by monitoring oral fluid concentrations among healthy controls, women with benign lesions, and those with ovarian cancer, found a significant elevation in CA 125 concentration among those with ovarian cancer. This suggests that the use of an oral fluid CA-125 assay can provide diagnostic sensitivity and specificity to that of a serum based assay.

Studies using different oncologic markers have also demonstrated that saliva testing may be useful in breast cancer detection measured by elevated levels of CA 15-3 and epidermal growth factor (EGF) receptor. The protein product of the oncogene c-erbB-2, also known as HER-2/neu, is elevated in the oral fluid of women diagnosed with breast cancer. The studies on this oral fluid marker are reliable and could ideally be used to monitor levels in patients during post-operative follow up (Tabak 2001; Li, St John et al. 2004).

1.7 Oral fluid and drugs of abuse

The use of oral fluid in monitoring of therapeutic and illicit drugs is in the process of replacing urine as a non invasive method of testing. With the easy passage of free-unbound drugs in oral fluid from serum, the monitoring of parental drugs can often be monitored with a short space of time from administration, the presence of cocaine and amphetamines can be measured in oral fluid prior to the availability in serum due to the basic nature of the drugs (caused by ion trapping which is dependent on the differences of oral fluid pH in comparison to blood) (Bosker 2009). The concentration levels of drugs and their metabolites can be effectively monitored within hours of administration and over an extended period of time that is mainly dependant of the rate of metabolism.

Barbiturates are sedatives used to treat convulsions and induce anaesthesia, they are subdivided into two groups' short acting amobarbital and long acting phenobarbital, and both are addictive drugs and can be used illicitly. Amobarbital levels in oral fluid can be measured up to 50 hours post administration (Inaba T 1975 , Dilli S 1980).

Cannabis is a psychoactive substance taken from Cannabis sativa; cannabis enters the body via smoking. Its levels in oral fluids are relatively low, which is ascribed to the smoking rather than active transport by saliva. In recent studies, it has been shown that it is possible to detect cannabis from oral fluid roughly 30 minutes after passive inhalation (Niedbala RS 2001).

Cocaine is a local anaesthetic and vasoconstrictor present in large amounts in the coca plant. In oral fluid, cocaine can be detected after all forms of administration, however mainly after smoking and

intranasal administration. The levels in saliva last roughly one hour after intake after which they are reduced to comparable levels found in blood (Jufer R 2006, Cone EJ 1997).

Nicotine is an alkaloid found within tobacco products. It is metabolized in the liver into cotinine and 3-hydroxycotinine. These two metabolites are measured in oral fluid to determine the intensity of smoking (and use of tobacco products) (Cone EJ 2007).

Codeine is a drug used for suppressing pain and coughing, it is metabolized by oxidation into morphine. Most often, it is administered orally, and can be used in combination with other drugs. A dosage of 60–120 mg of codeine is detected in oral fluid after 1 hour with a maximum concentration after 1.6–1.7 hours (Kim I 2002). The concentration of codeine in oral fluids is 3–4 times greater than its counterpart in plasma (Shirtcliff and Marrocco 2003).

Oral Fluid testing does have disadvantages in drug testing as drugs can cause hyposalivation (reducing sample volume) limiting sample volume. The concentration levels of drugs are lower in oral fluid in comparison to urine and are also open to contamination from food and bacteria within the oral cavity (Bosker 2009, Huestis 2004).

1.8 Overview

Oral fluid diagnostics and the use of non-invasive oral fluid collection devices have been used to monitor antibodies directed to viral insult and immunisation but also provide potential screening for localised/ systemic disease markers and substance abuse. This process can only be furthered by a better understanding of all oral fluid constituents and what they can tell us.

The developments in oral fluid collection and standardisation will help in the development of better screening methods and enable us to look for more elusive markers presented with this novel unique diagnostic matrix.

Chapter 2

The development of Seradate: an oral fluid collection device

2.1 Introduction

Our mission statement was to create a new oral swab that would allow collection oral fluid sample of higher OMT content to existing OFCDs. The name of our collector was termed Seradate; this was a combination of the words serum and transudate. These would be the components on interest that would be collected.

Normal oral fluid collection is conducted by drooling saliva into a tube; this method of collection is variable and user dependant. By using an OFCD oral fluid collections can be standardised for their use in assessments conducted in routine clinical, field-based and experimental applications.

Post sample collection procedures are also important as the oral fluid samples contain a valuable number of testable proteins, these are at risk of degradation by the presence of bacterial and saliva based proteases(Streckfus and Bigler 2002; Chevalier, Hirtz et al. 2007; Schipper, Loof et al. 2007). The effects of degradation and post sample processing of oral fluid will be reviewed in chapter 4.

There are numerous OFCDs and their individual methods of collection have shown properties of concentrating oral fluid samples prior to testing (Vyse, Cohen et al. 2001; Chevalier, Hirtz et al. 2007; Kozaki, Hashiguchi et al. 2009). Samples collected by an OFCD also appear less viscous and turbid in comparison to drooled oral fluid, making the testing of the fluid easier and reducing volume related errors in pipetting. The standardisation and collection of oral fluid has been investigated in literature,

systematic reviews by Crouch et al have highlighted a necessity for the standardisation of volume of oral fluid collected to remove ambiguity in final concentration of compounds in the collected samples (Crouch 2005).

Oral fluid is composed mainly of water and protein (the proteins present are composed of salivary based proteins, OMT and oral flora). Advances in proteomics and oral fluid protein profiling, have shown that OMT based markers can be successfully used in monitoring disease states within the body. The level of OMT outflow in normal healthy gingiva is relatively low and its levels are of serum transudate levels. An elevation in OMT and its protein content is evident in inflamed and periodontally challenged gingivae. In order to collect a sample of higher OMT protein content, we aim to use theories outlined in the literature that will exhibit a mixture of 2 definitions of OMT properties that are serum transudate and inflammatory exudate.

As the field of non-invasive testing is a platform for cost-effective screening at a large scale, it has a potential use for field based medicine and in low maintenance medical clinics. Marketed OFCDs use a method of collection, that involves swabbing of the gum surfaces and removal of the collector once saturated. The OFCD construct in its simplest form is a pad that is affixed to a plastic handle. The collectors vary in size and shape and their application is uniform.

The absorbent materials of OFCDS are subjected to a battery of tests that include their suitability to be placed in the oral cavity, and to collect and release oral fluid and its measurable compounds. The composition of the materials range from simple cotton based materials to laser treated sintered plastics. All

materials used show hydrophilic properties and have a high affinity for absorption and release of water and oral fluid alike. The suitability of the placement of the material within the oral cavity comes under user compliance; the material should not be too hard or abrasive in nature that may result in injury. The materials used all share an ability to absorb and release oral fluid, but the extent of release is usually governed by effective processing of the sample after collection and also the sensitivity of the oral fluid assay used. The retention of oral fluid in the material after processing may limit the availability of unbound proteins for testing.

2.2 Aims

The aims are to show the development of the Seradate OFCD from early concepts to working prototype. Each stage of development will be discussed and the reasons that led to the final development of the Seradate OFCD prototype.

The absorbent materials for the swabbing function of the Seradate will be reviewed. The materials all underwent tests for their ability to collect and release fluids and also their final fluid retention.

To simulate the collection of OMT-rich oral fluid, the materials were tested for collection and release of IgG of known concentration. IgG is predominantly present in low quantity in oral fluid and is regarded as a surrogate marker for OMT. The levels of IgG retention within each of the tested materials provides a percentage of un-testable content trapped within the material.

A questionnaire comparing the Seradate against current OFCDs will be used to show user compliance and highlight any potential limiting factors in the prototype OFCD.

2.3 Section A: Design and prototyping Seradate

Prerequisites of Seradate collection device

In order to create an OFCD that would fulfil the needs of adequate OMT-rich fluid collection, certain prerequisites were established as a guideline to completing our goal of creating an OFCD.

Simplicity

The collector should be self-explanatory for its intended use. The simplicity of design dictates its use as an oral applicator. The final design of Seradate should aim to achieve this.

Patient acceptability

The collector should be patient friendly and marketed as a non-invasive means of collection. The acceptance of the OFCD by the end user is of great importance.

Ergonomic design

The collector should be streamlined for its manner of use. The collector should not have any unnecessary attachments and its size and shape should adequately fit within the mouth of an adult.

Aesthetics

The collector should be pleasing to the eye as it is a potential commercial property. This point shares its basis with patient acceptability. The OFCD should be non-threatening and should be visually directive of its non-invasive use.

Health and hygiene

The collector should be sterile and absorbent materials used should be inert and of food safe quality. This is of paramount importance as with all medically related instruments, it should conform to basic hygiene.

Sample volume

The collector should collect a greater volume of OMT that is comparable to commercially available OFCDs. This is a potential marketing point. As OFCDs provide easier and less messy method of collection, the level of fluid collected is a lot lower in comparison to its invasive counter parts. By collecting a greater volume, this will reduce the requirement for repeat testing or secondary sample collection.

Sample integrity

Post collection the OFCD should be stored appropriately prior to analysis. Sample integrity will be reviewed in more detail in chapter 5. Oral fluid sample storage conditions post collection is a key component, if left in non-standardized conditions this often results in sample degradation.

Low production cost

The cost of production should be low as possible to improve commercial viability of the OFCD. An overall lower production cost would yield a greater profit should the OFCD be marketed.

Seradate: Stage 1 concepts

The toothbrush concept

The initial concept of the Seradate resembled that of a standard toothbrush. The design was chosen to induce familiarity to the user and its proposed use would be self-explanatory (figure 2.1). The functional use of a toothbrush is to clean the surface of the teeth by a rubbing/ abrasion method. This procedure could potentially be mimicked to induce partial trauma to the gum surfaces to promote the release of OMT, this form of collection is currently used by many OFCDs (such as Orasure and Oracol). The toothbrush handle shows aesthetic and ergonomic design this would allow the collector to be held comfortably within the oral cavity during sampling. The design of the toothbrush is ideally suited for an OFCD but resembles the properties of current marketed devices. The manner of oral fluid collection would be similar to that of Orasure and Oracol OFCD.

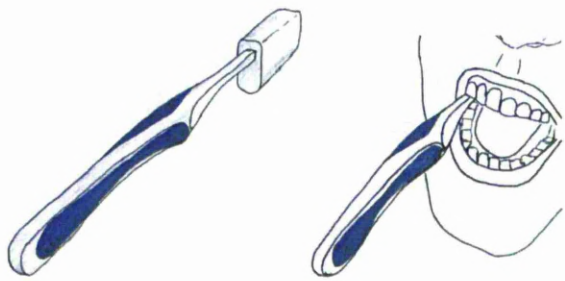

Figure 2.1 The toothbrush concept. The conventional head has been replaced by an absorbent pad. The handle will help induce familiarity with oral fluid collection.

The suction concept

The toothbrush concept could be supplemented with suction; this could potentially promote the uptake of a sample volume that is far greater than that achievable by swabbing. The limitations of swabbing are sample volume collected is dictated by the amount of material used. The Integration of this method with abrasion of the gum surfaces could increase OMT release and allow a greater final sample volume to be collected (figure 2.2). The level of suction required for the procedure would be variable and subjected on an individual basis this would include age and current gum health. The methods of suction that can be used would include manual suction by hand pump or automatic suction using a motorised pump (similar to that used in an electronic toothbrush). The collected sample can then be siphoned into a separate container.

Figure 2.2 A motorized addition to the toothbrush concept in figure 3.1. The handle is hollow to allow easy passage of collected oral fluid that can be siphoned into a separate container.

Stage 1 design

The stage 1 design for the prototype resembled the concept below (figure 2.3). The proposed use of suction and hand pump would not be viable for on-field screening, and the additional cost of the unit would greatly reduce its potential marketable value. The design would therefore mimic that of current marketed OFCDs, thus giving it the ability to collect an oral fluid sample that exhibits OMT properties. As the compound of interest is OMT a more site specific design would be better suited to the collection of OMT, a new concept would be required that would help induce the release of OMT and its effective collection.

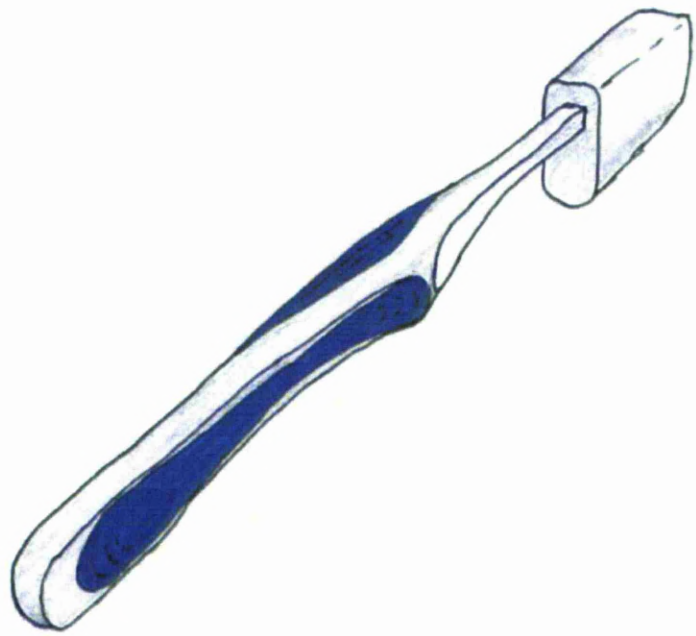

Figure 2.3 Prototype design for Seradate OFCD

Seradate: Stage 2 concepts

The dual mouth guard concept

The outflow of OMT is localized around the gingival crevice and its surrounding surfaces. This led to the suggestion of shell-like construct to cover the teeth and gums, as OMT is present in all oral fluid samples its final concentration is lowered by saliva dilution. The closest similarity to this idea is seen in the dual mouth guard, this covers both the upper and lower portions of the gums. This will enable collection from two sites by a single collection, thus reducing the requirement for multiple sample collection for testing purposes. The primary function of the mouth guard is to protect the mouth in occasions of blunt trauma induced by various sporting activities including boxing, football etc. The mouth guard encloses the teeth and gums from the rest of the oral cavity. The isolation of a region within the oral cavity would permit specific collection resultant in enriched OMT collection. The manner of collection will involve the mastication process (biting down) on the mouth guard surface against the teeth and gums, to induce mild trauma to the gingival surfaces, leading to the release of OMT.

The size of the dual mouth guard would affect the collection purposes with potential user mediated contamination. To make use of this concept a selected portion of the oral cavity would be targeted and not the entire oral cavity. The two suggested regions of placement were the front teeth and the molar/premolar region. The regions were chosen to maximise both comfort and ease of use. The final area for collector placement was decided to enclose the molar/premolar region as this would be more comfortable to the user.

Stage 2 design

The design was constructed with the aid of Russell Bland (freelance designer).The design has taken its inspiration from the dual mouth guard concept (figure 2.4). The measurements for the device head were taken from different sets of dentures provided by the University of Liverpool Dental School. Accurate measurements were made using callipers to find approximate dimensions of the sampling head to allow comfortable placement in the mouth. The handle design was taken from the toothbrush to induce familiarity and features a separation point. This is required to separate the head into an adequate tube minimising contamination of the collected sample and also protects against contact via other uses.

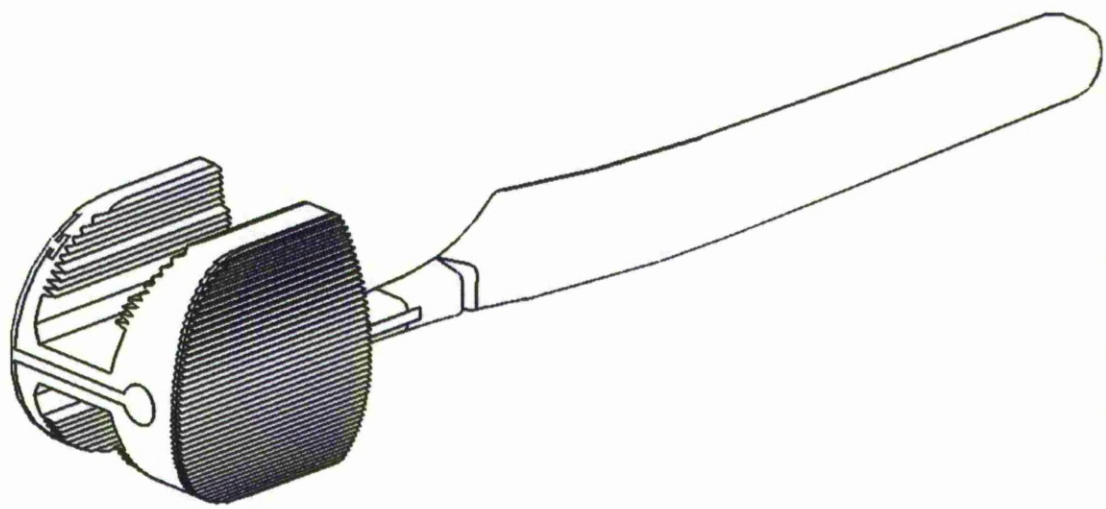

Figure 2.4 Stage 2 Seradate design

Patent number GB 0802710.4 (schematic drawn by Russell Bland)

The proposed composition materials for the device are two separate parts. The head portion would be composed of a flexible soft hydrophilic treated polymer either in the form a sintered plastic or fibre (polyethylene, polyurethane, polyolefin) as this would be the point of primary contact for the capturing OMT. The texture and consistency of the material would be evaluated to ensure comfort to the user. The composition of the exterior portion would use a hydrophobic treated polymer sintered plastic or fibre (polyethylene, polyurethane, polyolefin) to repel any excess saliva produced within the oral cavity.

The head of the device has an H-shaped design to accommodate both the upper and lower gums to allow for greater sample collection.

The Inner head portion

The inner head portion would feature protrusions for the following reasons (figure 2.5)

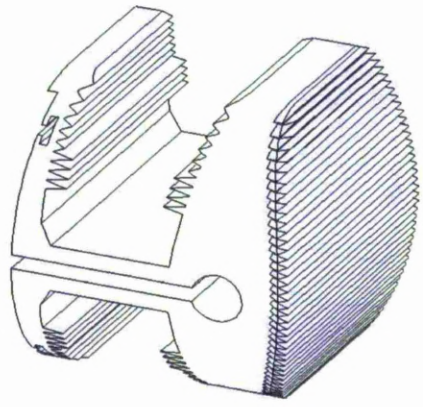

Figure 2.5 An outline of the head of the stage 2 Seradate

1. An increase in surface area to contact area ratio between the gum surfaces and the absorbent pad. To expose material to the gum surface in a shorter period of time.

2. The protrusions would allow for adhesion via contact of teeth to the surrounding material. This has the benefit of ensuring that the device shows minimal lateral movement and will allow standardised use amongst a variety of users.
3. The protrusions will engage the gingival surface through the actions of biting. This stimulation will lead to the release of OMT.

The head of the device is composed of an upper and lower portion. To allow samples to be taken from both upper and lower portions of the oral cavity reducing the need of multiple samples to be taken. The cross-section of the device has a bite plate to engage the gingival sulcus of the buccal cavity and is composed of a hydrophilic polymer.

Stage 2 prototype

The final design was made into a prototype using a composite of high density polyethylene. It was evident that even with the relevant dimensions being made available from a variety of dentures taken from a normal adult mouth; the head of the construct was too large and cumbersome to be easily placed within the oral cavity in a comfortable manner, as both upper and lower gum surfaces were engaged, this limited its proposed use. One of the main flaws of the design was not taking into consideration the opening of the mouth, as the head of the collector only took into account average sizes of teeth and gum surfaces. The concept of a dual mouth-guard is promising and may work if the size of the head is reduced.

Seradate: Stage 3 concepts

Mouth guard concept revisited

Using the concept described at stage 2, the idea of shielding the area of collection was retained (region specific for OMT collection) and the design concepts were re-drafted in collaboration with the Department of Engineering at the University of Liverpool (two students from the school of engineering and design were recruited and supervised throughout this procedure). This produced a design that could be finally tested. Figure 2.6 shows how the idea of using mastication and abrasion was re-assessed. The idea was shifted from targeting the upper and lower gums surfaces to focussing on the lower gum region to allow for greater comfort and access of the collector within the oral cavity, the stages of design can be seen in figures 2.7 and 2.8.

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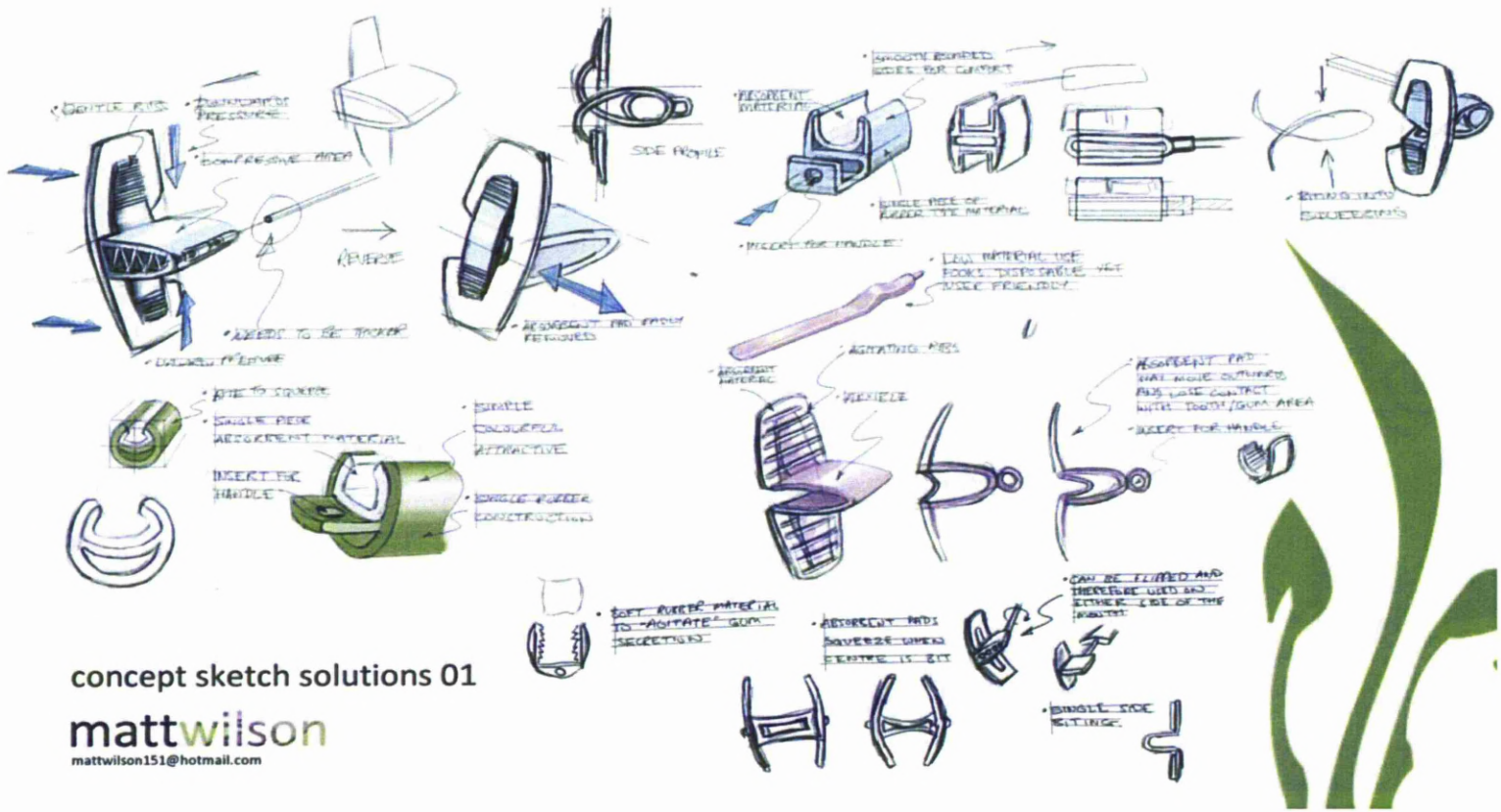


Figure 2.6 Stage 3 concept design (Sketches drawn by Matt Wilson. University of Liverpool)

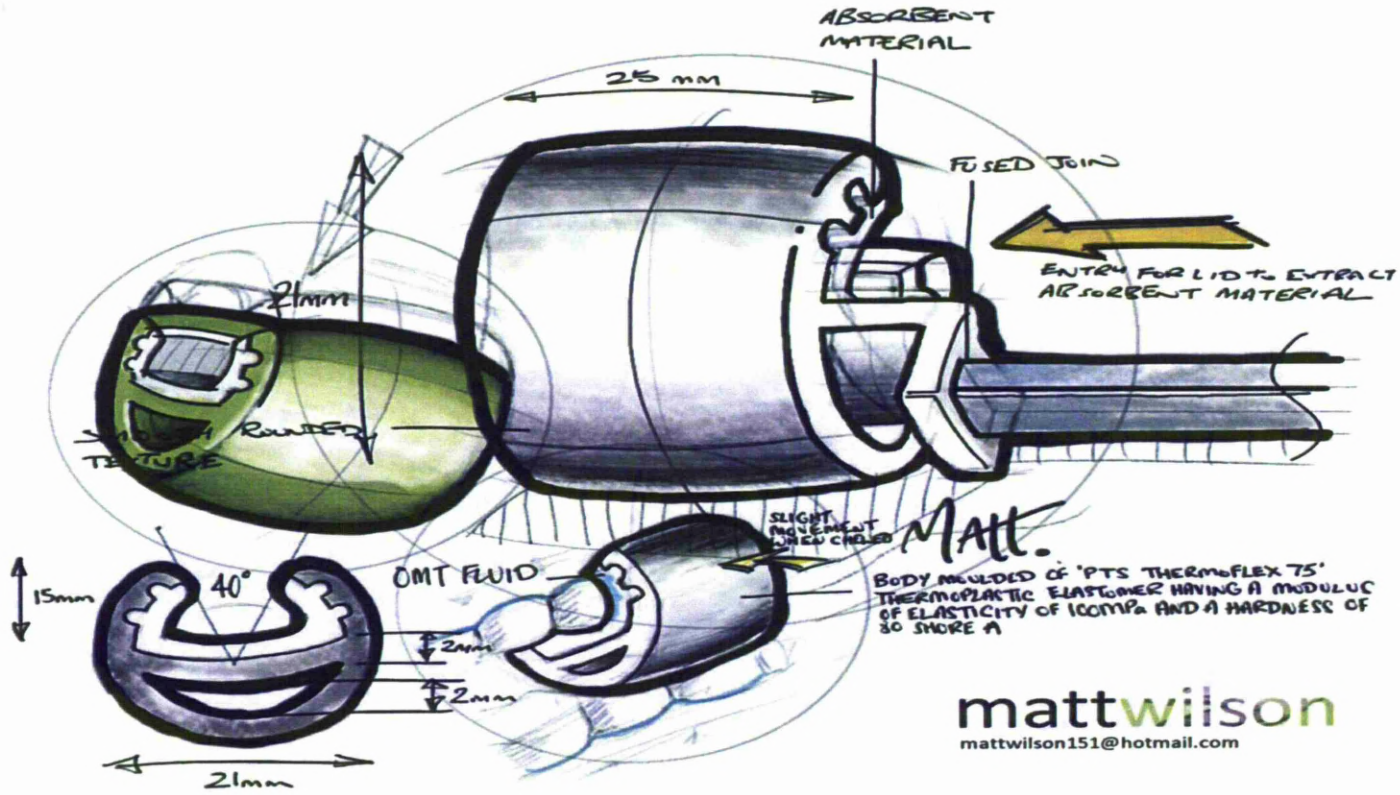


Figure 2.7 Stage 3 concept design (Drawn by Matt Wilson. University of Liverpool)

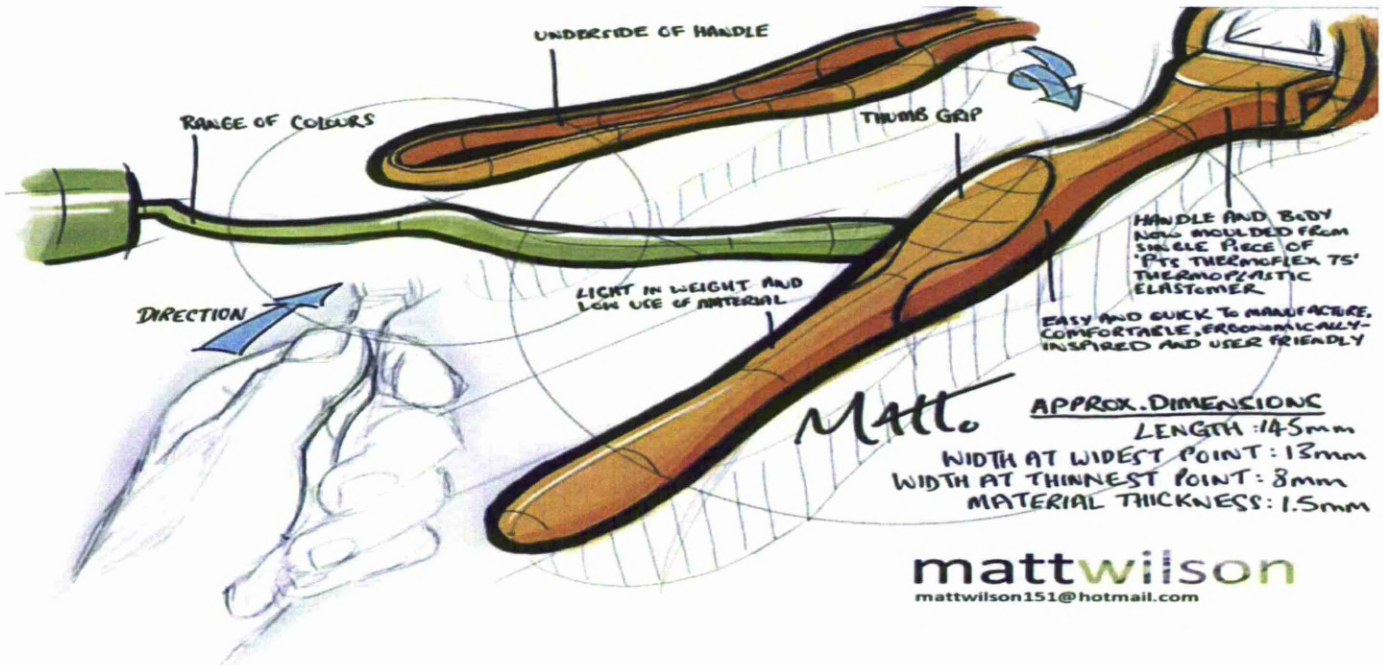
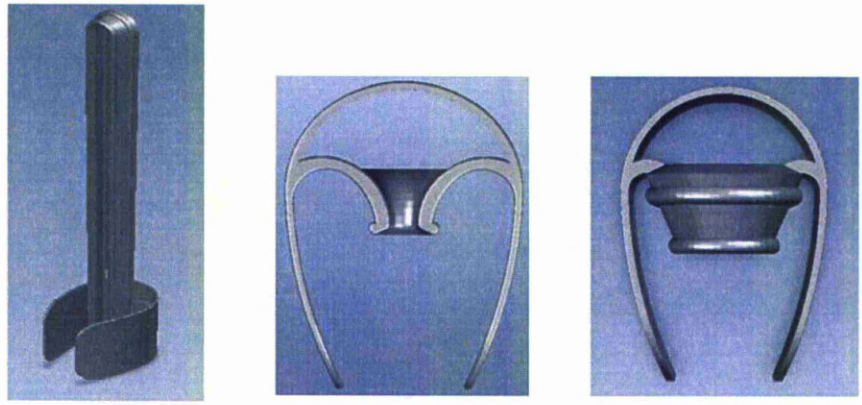


Figure 2.8 Stage 3 concept design (Drawn by Matt Wilson. University of Liverpool)

Stage 3 Design

The collection site of the Seradate would use the lower (inferior) gum surface as its main area of sample collection. As the proposed method of collection involves both abrasion and mastication the shielding on the Seradate head would reduce further dilution of the collected sample by saliva (figure 2.9).



A B C

Figure 2.9 Seradate stage 3 design. (A)Longitudinal view (B) Anterior View (C) Posterior View. The designs were produced using ProEngineer software (By Abadur Rohman and Matt Wilson. University of Liverpool

The targeted regions of the lower gums would provide adequate shielding from parotid secretions that would be released upon inducing the mastication process. The handle design was altered to allow more flexibility in the handling of the collector as the rigid nature of the original design would not allow easy manipulation of the collector once placed into the oral cavity.

Stage 3 Prototype

The prototype of the Seradate was made out of flexible polyurethane to allow for easier biting action (Figure 2.10).

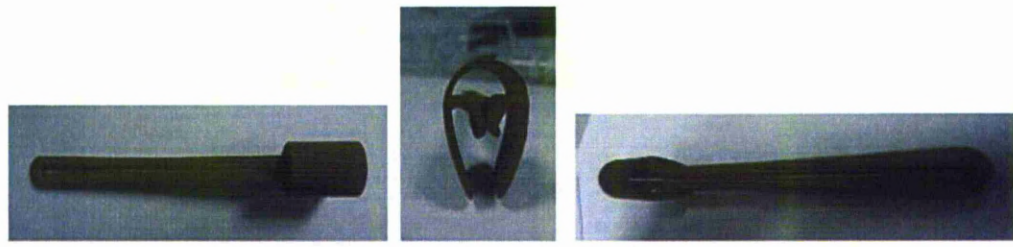


Figure 2.10 The final images of Seradate collection device

The collector was manufactured by MNL (Malcolm Nicholls Limited). The mechanism of action would be to bite down on the head to cause the lateral sides of the collector to protrude outward when pressure is applied and contract when pressure is released this continuous agitation of the gum surface would lead to partial abrasion of the gum surfaces and facilitate the release of OMT. The proposed movement of the device head would form a temporary capsule around the gum region of interest and the absorbent interface would be shielded from parotid secretions, this would reduce OMT dilution of the final collected sample. The handle design was changed from the initial toothbrush handle to accommodate for flexibility. The absorbent material was placed under the lips of the device. The Seradate prototype was tested alongside current marketed OFCDs for its ability to collect and release oral fluid (chapter 3) and its ability to release oral fluid components (Chapter 3, 5, 6).

2.4 Section B: Seradate absorbent material tests

Having developed a testable prototype we looked at various materials that could be adequately placed within the oral cavity that are able to collect and release oral fluid. The test materials used in our assessment were obtained from Porex (Germany) and Filtrona Fibertec (Germany) (Table 2.1). The materials chosen for use were specifically engineered for oral fluid collection and all showed characteristics of wicking. Wicking is also known as Capillary attraction, or capillarity, it is the ability of a fluid to flow through narrow spaces without the assistance of, and in opposition to external forces such as gravity. This occurs because of inter-molecular attractive forces between the fluid and solid surrounding surfaces and is a combination of surface tension (which is caused by cohesion within the liquid) and adhesive forces between the fluid and material causing the fluid to rise.

Each of the test materials were laser treated to induce optimal hydrophilic properties.

The tested materials were sintered polyethylene and polyolefin. Sintered plastics are characterized by high purity, uniformity and stability; this lowers its chances of falling apart in the mouth. Sintered plastics can formed to specific pore sizes which would be very useful in oral fluid collection, so as to reduce sample concentration errors.

Polyolefin are fiber-based materials that do not provide specific porosity as witnessed in sintered plastics. Polyolefin are not as coarse and softer in comparison to sintered plastics and can be more easily molded to fit specific shapes.

Each of the materials was tested for their ability in the retention and release of water/saliva and their final percentage retention after processing. To test the materials for monitoring oral markers each material was also tested for the uptake and release of IgG (A surrogate marker for OMT).

Material	Composition	Comfort*	Supplier
BNW 1	Bonded polyolefin fibres hydrophilic surface finish	Good	Filtrona Fibertec
BNW 2	Bonded polyolefin fibres hydrophilic surface finish	Good	Filtrona Fibertec
D3527G	D3727g bi-component Hydrophilic Nylon/ PET	Good	Filtrona Fibertec
XS-1	1.5 mm thick 20-60 µm hydrophilic sintered plastic	Poor	Porex
XS-2	1.0 mm thick 20-60 µm hydrophilic sintered plastic	Poor	Porex
XS-3	1.0 mm thick 40-100 µm hydrophilic sintered plastic sheet	Poor	Porex
XMF-1	sintered plastic sheet	Poor	Porex
T3	sintered plastic sheet	Poor	Porex

Table 2.1 Absorbent materials tested for use in the Seradate OFCD prototype.

*The level of comfort was measured via placement in mouth.

2.4.1 Materials & methods used to test materials

Standardising absorbent materials used

To standardize our testing of the absorbent materials, each tested material was cut to measure 3cm*3cm square, the thickness of each material accounted for and material was weighed (dry weight). The materials were placed individually into 10 ml of distilled water to ensure complete saturation and final-weight recorded (final mass). The time period allowed for complete saturation was approximately 2 minutes; this was to ensure that all materials tested were fully saturated. The volume collected was determined as follows

Volume collected (µl) = Final mass (µg) – Dry weight (µg)

The percentage of distilled water/oral fluid retained in tested materials

Alongside volume collection, each material was tested for water/oral fluid retention. The saturated materials were spun down via centrifugation and weighed (final weight).The final retention was expressed as a percentage. The level of retention of fluid in the material was calculated as follows:

$$\text{The percentage of water retained (\%)} = \frac{\text{Final weight (mg)}}{\text{Dry Weight (mg)}} * 100$$

Total IgG released by the tested materials

To measure the levels of IgG released by the test materials, the sample materials were placed in a pool of known concentration of IgG (150 ng/ml) for a period of 2 minutes. The concentration of IgG released by each material was measured using Human IgG Elisa Quantitation Kit Bethyl Laboratories Inc (Montgomery, USA) according to manufacturer's instructions. 96 well High Bind flat bottomed plates (Costar Corning Lifesciences, UK) were coated with goat anti-human affinity antibody (Primary Antibody, Bethyl laboratories Inc, USA) .The number of wells used was determined by assay standards, controls and the total number of samples tested (either duplicate or triplicate). 1 µl of capture antibody diluted with 100ul of coating buffer (0.05 M Carbonate-Bicarbonate Sigma Aldrich, UK) administered to each test well. The plate incubated at 21°C for 60 minutes or overnight at 4°C.

The coating buffer was removed by a 3 step wash using a Tecan Columbus plate washer (Austria) with PBS 0.05% Tween 20. Each step of the wash involves washing of the wells and aspirating the wells, this process is repeated a total of three times within the 2-step wash process, the plates are then dried by tapping onto blue roll. 200 µl of blocking solution (Phosphate buffered saline, 1% BSA, pH 8.0) was added to each well. The plate was incubated for 60 minutes at 37°C. After incubation the blocking solution was removed by 3 step wash (as previously described).

The assay standards used human serum as reference (final serum concentration 1000 ng/ml according to manufacturer's guidelines). 100 µl of sample diluent (Phosphate buffered saline 1% BSA, 0.05% Tween 20, pH 8.0) was added to each well. 100 µl of dilute standard was added to the first well, mixed and 100 µl removed

and added to the second well, the process was repeated for the next 7 wells. This produced a standard curve ranging from 1000 ng/ml - 7.8 ng/ml. Oral fluid samples were added these were diluted to optimal concentration to specific wells (1:100 dilution from neat). The plates were then incubated at 21°C for 60 minutes.

The samples were removed by 5 - step wash (This is a similar process to the 3 step wash). Secondary HRP detection antibody was added to each test well, this was diluted from stock (1:50,000).100 µl of secondary HRP-detection antibody was added to each well and the plate incubated for 60 minutes. After Incubation the secondary HRP-detection antibodies were removed using a 5 - step wash and 100 µl 3, 3', 5, 5'-Tetramethylbenzidine (TMB, Sigma Aldrich, UK) was added to each well. The plate was sealed, covered and placed on a rotating platform for colour development. The development time was between 5-10 minutes, the reaction was stopped by adding 100 µl 4N H_2SO_4 to each test well. The plate was analysed at 450 nm on a spectrophotometer (J Bio LP 400). The sample Immunoglobulin concentrations were calculated from a standard curve using Microsoft Excel (for a detailed list of materials used please refer to appendix B).

The percentage of IgG retained within the tested materials

To compare the levels of IgG retention within the tested materials, the percentage of retention was calculated.

$$\text{The percentage of IgG retained (\%)} = \frac{\text{original concentration (ngml)}}{\text{final concentration(ngml)}} * 100$$

2.4.2 Assessment of materials used in the Seradate

Standardising absorbent materials used

The materials collected different volumes of distilled water (figure 2.11). A significant increase in collection was seen with D3727g in comparison to all tested materials which collected 4000µl. Marginal significance was expressed between BNW1 and BNW 2. XMF-1 each collects approximately 2000 µl - 2500 µl.
Each BNW 1, BNW2 and XMF-1 showed a significant increase in collection in comparison to XS-1, XS-2, XS-3 and T3. The test materials XS-1, XS-2, XS-3 and T3 these showed marginal significant collection each collecting an average of 500 µl.

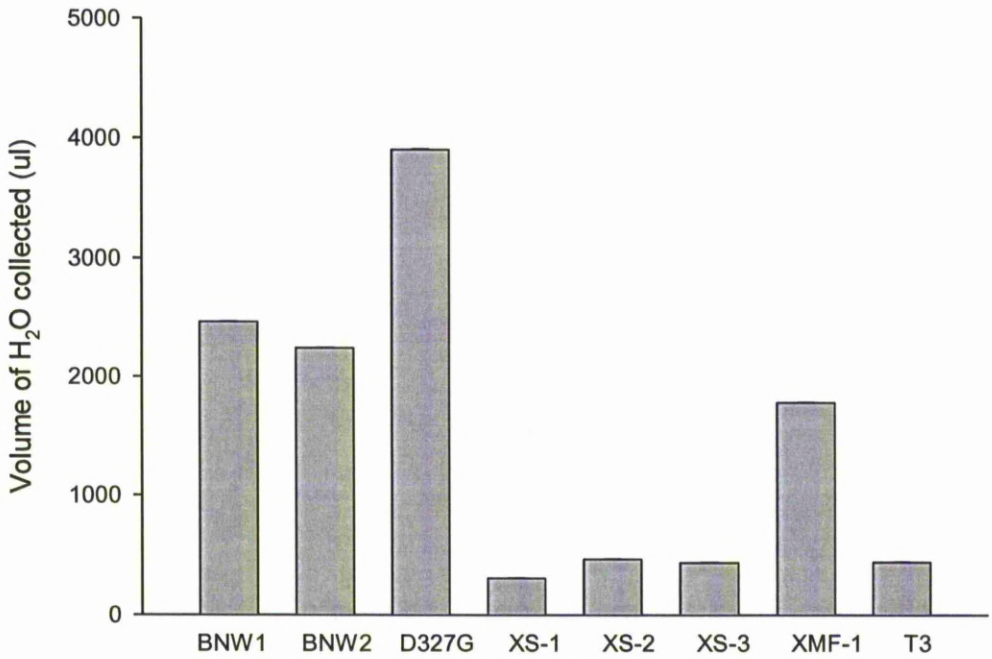


Figure 2.11 The volume of H_20 collected by various novel biomaterials. The volume collected was measured in µl. The SEM was too small to be seen in the chart. n =5

The percentage of distilled water retained in tested materials

The materials retained different percentages of distilled water (figure 2.12). D3727g and XS-1 showed significant retention at approximately 4% in comparison BNW 1, BNW2, XS-2 XS-3 and XMF-1. BNW 1, BNW2, XS-2 XS-3 and XMF-1all showed comparable retention levels of approximately 2%. T3 showed the most significant retention in comparison to all tested materials at approximately 30%. This is a range of 7.5 - 15 fold increase in comparison to its tested counterparts.

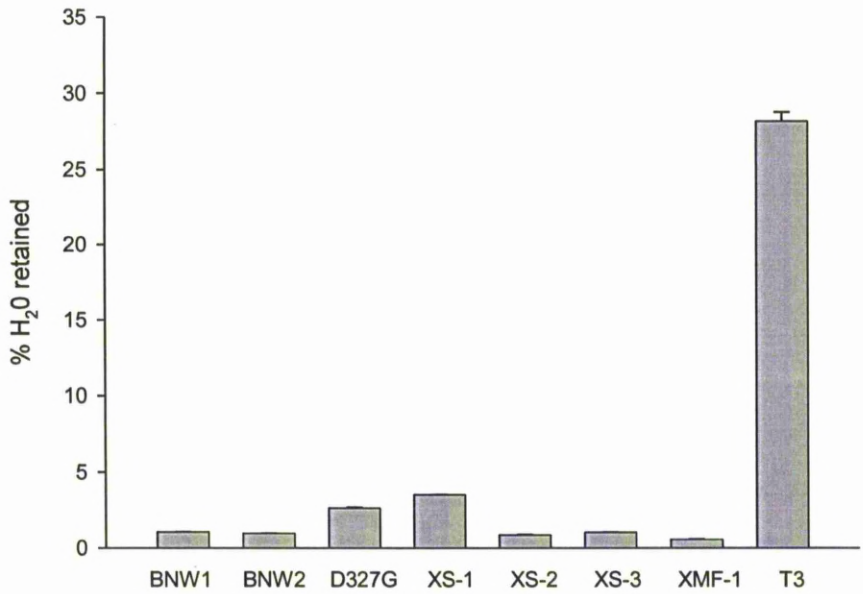


Figure 2.12 The percentage of H_20 retained within biomaterials The error bars show +SEM. n =5

Testing materials for collection of oral fluid

The materials collected different volumes of oral fluid (figure 2.13). A significant increase in collection was seen with D3727g in comparison to all tested materials which collected 4000 µl. Marginal significance was expressed between BNW1 and BNW 2. XMF-1 each collects approximately 2000 µl- 2500 µl. Each BNW 1, BNW2 and XMF-1 showed a significant increase in collection in comparison to XS-1, XS-2, XS-3 and T3. The test materials XS-1, XS-2, XS-3 and T3 these showed marginal significant collection each collecting an average of 500 µl.

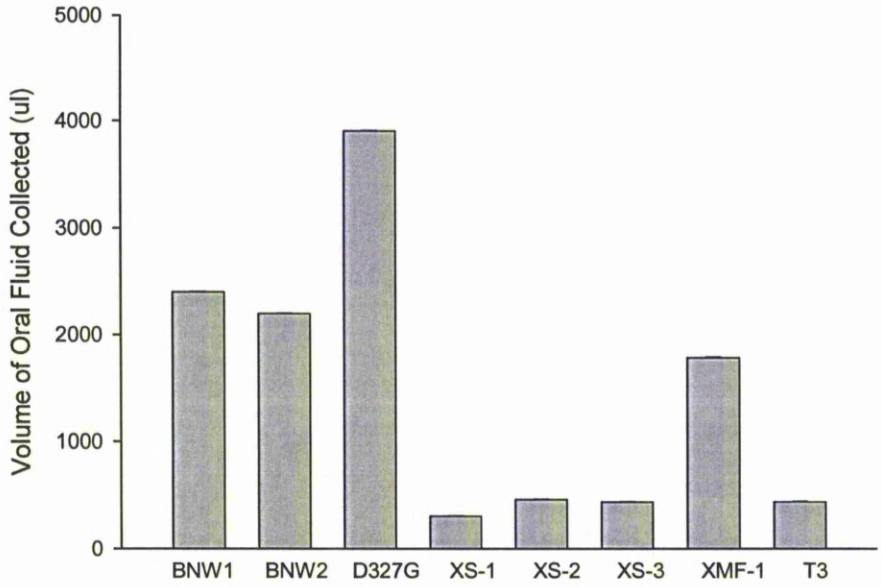


Figure 2.13 The volume of oral fluid collected by various novel biomaterials. The volume collected was measured in µl. The SEM was too small to be seen in the chart. n =5

The percentage of oral fluid retained in tested materials

The materials retained different percentages of oral fluid (figure 2.14). D3727g and XS-1 showed significant retention at approximately 4% in comparison BNW 1, BNW2, XS-2 XS-3 and XMF-1. BNW 1, BNW2, XS-2 XS-3 and XMF-1all showed comparable retention levels of approximately 2%. T3 showed the most significant retention in comparison to all tested materials at approximately 30%. This is a range of 7.5 - 15 fold increase in comparison to its tested counterparts.

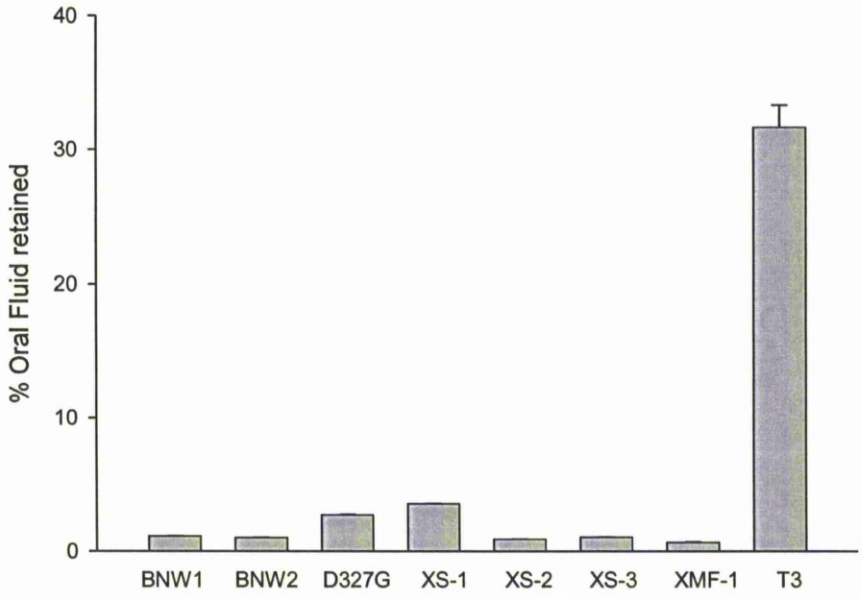


Figure 2.14 The percentage of Oral Fluid retained within the materials The error bars show +SEM. n =5

Total IgG released by the tested materials

The total IgG released by each material from the original concentration of 150 ng/ml was assessed (figure 2.15). BNW-1, D3727G and XS-2 released marginally significant levels of IgG ≈100 ng/ml, and were significantly higher in comparison to BNW-2, XS-1, XS-3, XMF-1 and T3.

BNW-2, XS-1, XS-3 and XMF-1 released comparable levels of IgG ≈90 ng/ml and were significantly higher in comparison to T3.

T3 released approximately 80 ng/ml of IgG from the initial concentration of 150 ng/ml; the material exhibited a significantly lower level of release in comparison to all tested materials.

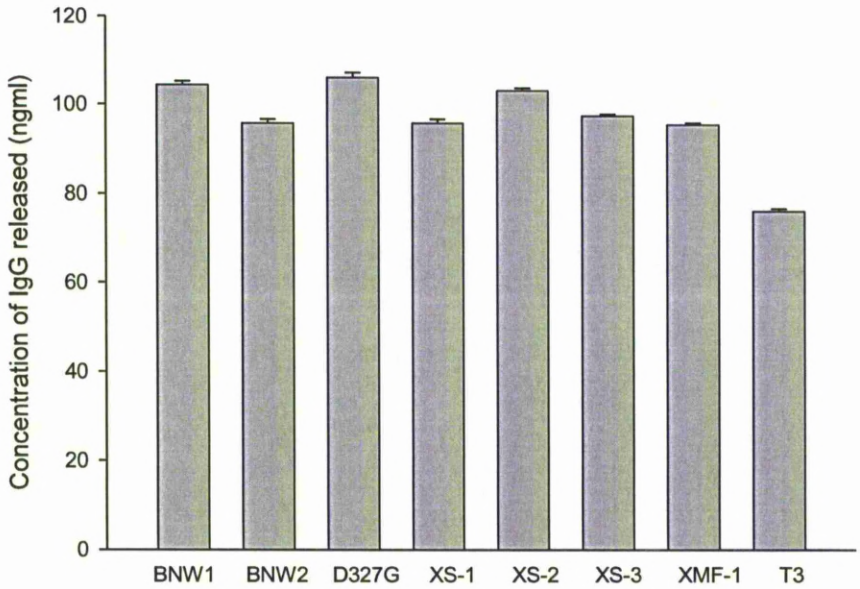


Figure 2.15 Total IgG released from known IgG pooled sample (150ngml). The error bars show ±SEM. n =5.

The percentage of IgG retained within the material

In figure 2.16 the data shows no significant differences between D3727g, BNW1 and XS-2 in retaining approximately 30% IgG. Marginally significant differences were also seen between BNW2, XS-1, XS-3 and XMF-1 in retaining approximately 35% IgG. T3 expressed the most significant difference in the level of IgG retention compared to all materials tested at a retention level of 50 % IgG.

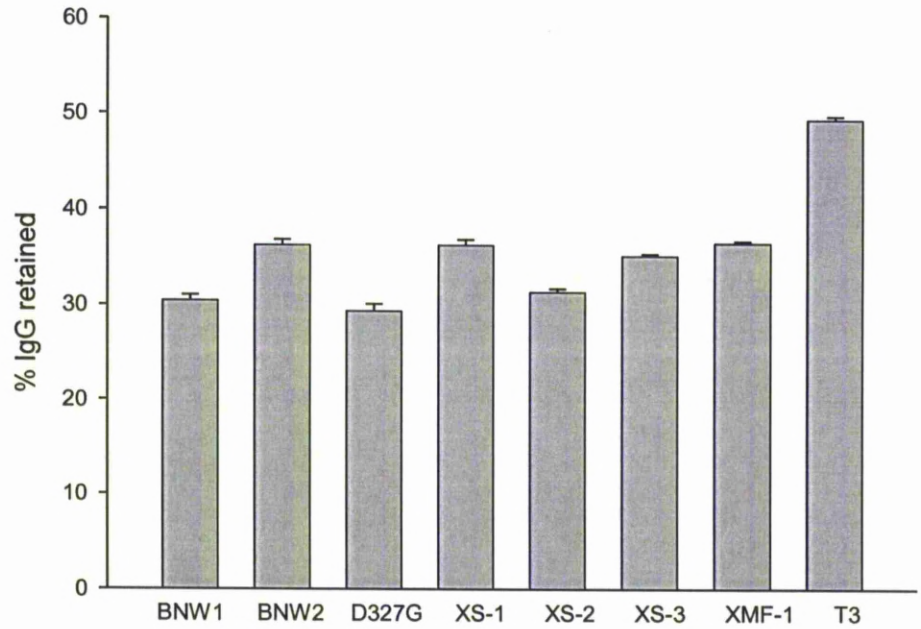


Figure 2.16 The total percentage of IgG retained within tested materials. The error bars show ±SEM. n =5.

2.5 Section C: User compliance of Seradate OFCD prototype with other collection methods

User compliance is of great importance as OFCDs are marketed for their ease and self-directed use. To examine the compliance of the Seradate a questionnaire was taken from volunteers within the study cohorts to compare the Seradate against other methods of oral fluid collection (see appendix for questionnaire and informed consent forms).

The questionnaire was subdivided into the following categories

- Aesthetics
- Ease of use
- Comfort
- Overall experience

Each volunteer was also asked to confirm their smoking status and dental hygiene schedule.

Each question was assessed on a likert scale of 10 (1= Very poor-10 = excellent). A rating of 5 was deemed as user acceptance.

For further feedback on each of the collected methods, volunteers were also asked to provide written feedback on each of the collection methods to evaluate their overall experience (refer to appendix A for questionnaire, informed consent and information sheet and appendix C for individual feedback questionnaires)

Aesthetics

Aesthetics is a measure of first impressions and visually appealing aspects of the OFCDs (figure 2.17).

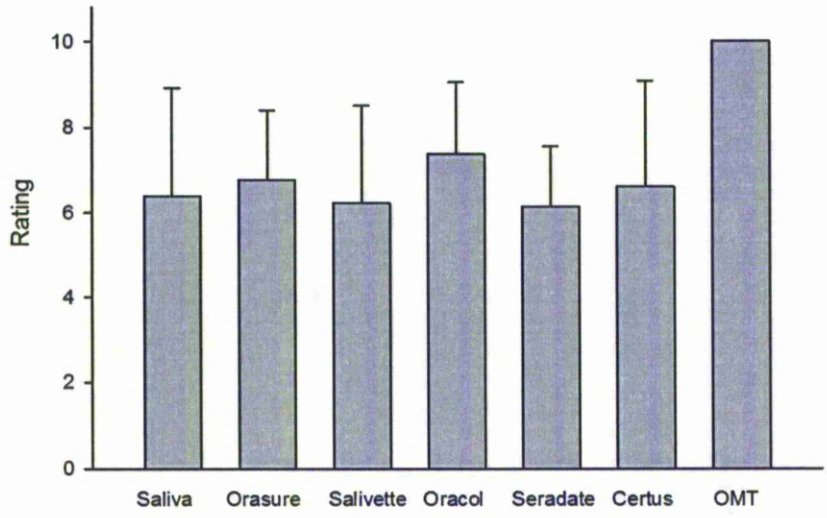


Figure 2.17 Volunteer ratings on aesthetics of oral fluid collection using different OFCDs. Rating scale of 1-10. 1= Very Bad 10=Excellent. Bar height shows SEM n=35

Saliva collection functioned as a control. There were no significant differences seen between the Orasure, Salivette, Seradate and Certus in comparison to the control (drooled saliva) as each collection method expressed a level of 6 and higher within the test cohort. The Oracol showed a greater level of acceptance at 7 whilst the collection of OMT by filter strip method was unanimously liked overall with a rating of 10. Individual feedback showed that 14 out of the 35 participants (40%) found saliva collection none

appealing followed by the Seradate (29%), Orasure (9%) and Oracol (9%). There was no dislike of the Certus. As acceptance for each of the test methods was set at 5, each of the collection methods in terms of aesthetics overall was satisfactory to all users.

Ease of use

The ease of use was to check the simplicity of each collection procedure after being provided with written and verbal instruction (figure 2.18).

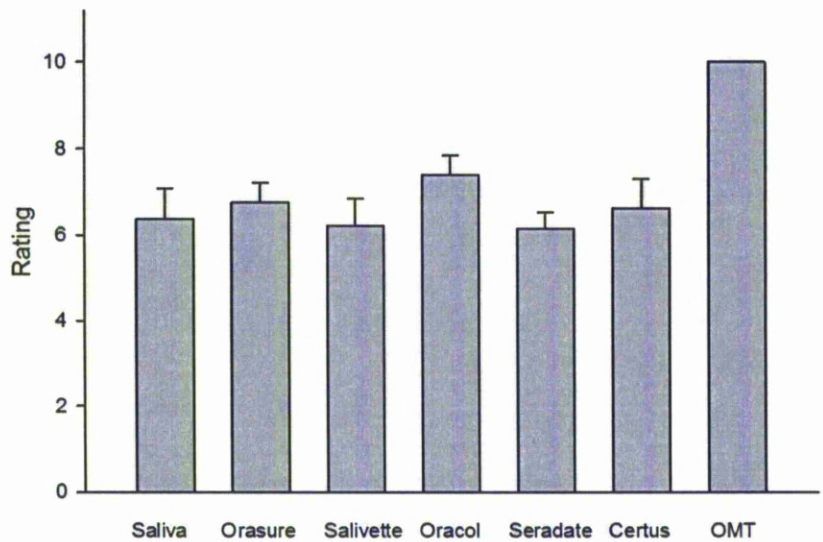


Figure 2.18 The rating on ease of use on oral fluid collection using different OFCDs. Rating scale of 1-10. 1= Very Bad 10 =Excellent. Bar height shows SEM n=35

Saliva collection functioned as a control. There were no significant differences seen between the Orasure, Salivette, Seradate and Certus in comparison to the control (drooled saliva) as each expressed a level of 6 and higher. The Oracol should a greater

level at 7 whilst the collection of OMT by filter strip method was unanimously liked overall with a rating of 10. Individual feedback showed that 10 out of 35 participants (29%) found the Seradate uncomfortable followed by the saliva collection (14%), Salivette (6%), Oracol (6%). There was no dislike of the Certus .As acceptance for each of the test methods was set at 5 each of the collection methods in terms of ease of use overall was satisfactory to all users.

Comfort

The comfort for each collection method was a measure of any pain or discomfort derived from each of the collection procedures (figure 2.19).

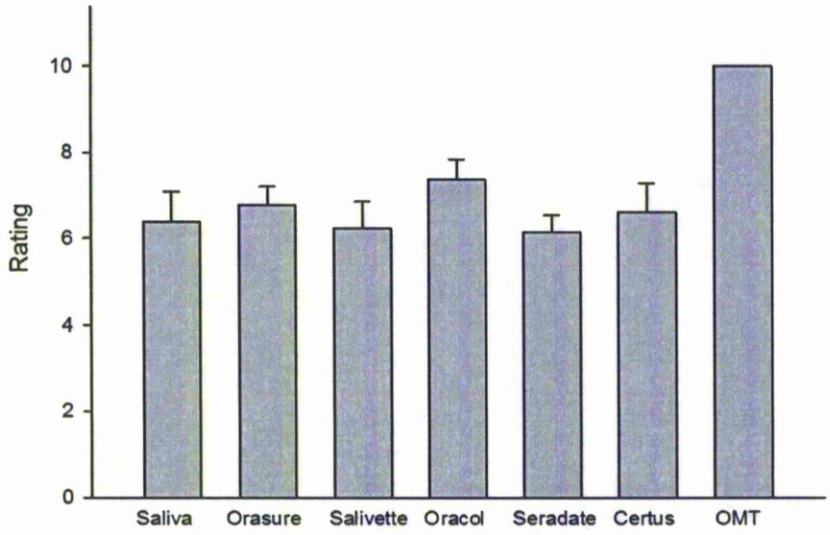


Figure 2.19 The rating on comfort of oral fluid collection. Rating scale of 1-10. 1= Very Bad 10 =Excellent. Bar height shows SEM n=35

Saliva collection functioned as a control. There were no significant differences seen between the Orasure, Salivette, Seradate and

Certus in comparison to the control (drooled saliva) as each expressed a level of 6 and higher. The Oracol should a greater level at 7 whilst the collection of OMT by filter strip method was unanimously liked overall with a rating of 10. Individual feedback showed 13 out of 35 participants found the Seradate uncomfortable to use (37%) followed by Salivette (23%), Orasure (20%), saliva collection (17%), Certus (14%) and Oracol(6%). As acceptance for each of the test methods was set at 5 each of the collection methods in terms of comfort was satisfactory to all users.

Overall experience

The overall experience was used a universal measure to encompass all previous aspects of the questionnaire and whether the participants were willing to do a repeat procedure if the need arose (figure 2.20).

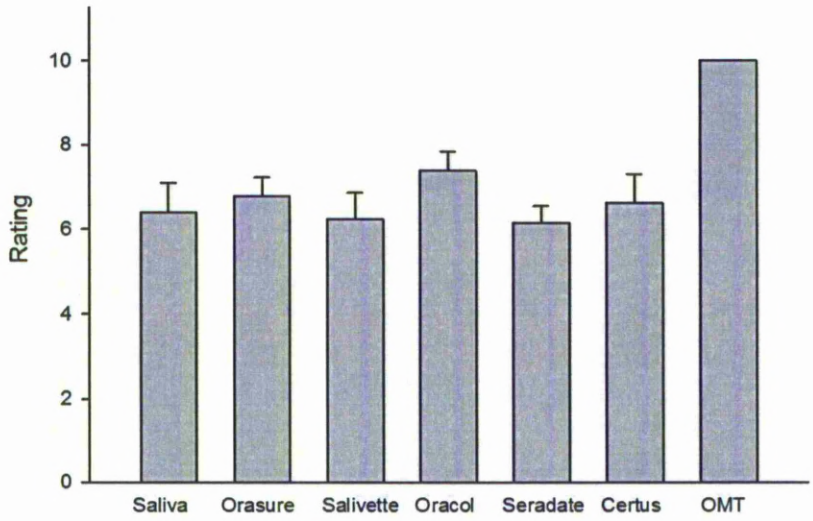


Figure 2.20 The rating on overall experience of oral fluid collection. Rating scale of 1-10. 1= Very Bad 10 =Excellent. Bar height shows SEM n=35

Saliva collection functioned as a control. There were no significant differences seen between the Orasure, Salivette, Seradate and Certus in comparison to the control (drooled saliva) as each expressed a level of 6 and higher. The Oracol should a greater level at 7 whilst the collection of OMT by filter strip method was unanimously liked overall with a rating of 10. Individual feedback showed that 10 out of the 35 participants disliked the overall experience of using the Oracol (29%) followed by saliva collection (23%), Salivette (14%), Orasure (14%), Certus (9%) and Seradate (3%).As acceptance for each of the test methods was set at 5

each of the collection methods in terms of overall experience was satisfactory to all users.

Feedback for collection procedures

Individual feedback was taken from each test individual and summarised (table 2.2). As the questionnaire showed that the OMT collection procedure (both inter and intra crevicular collections) was unanimously accepted as the collection method of choice. Its disadvantages lay in the requirement of a dentist for collection.

Drooled saliva served as the control for the study, this was commended on its ease of collection but was least liked and deemed unpleasant in comparison all other methods of collection.

Orasure OFCD was commended of ease of use and comfort when placed into the mouth; however the salty after taste was slightly unpleasant but not scrutinized.

Salivette OFCD was commended on easy positioning in the oral cavity (superior or inferior to the tongue). It did have an unpleasant after taste and collector did feel awkward in the mouth.

Oracol OFCD was commended on its ease of use and comfort. It did leave a slight after taste but was not unpleasant.

Seradate OFCD was commended on being comfortable around the gums; the collector was stated as too large for some volunteers and slight discomfort felt by one user. The pads if smaller would have fit better within the construct.

Certus OFCD was commended on its aesthetics, it looked good and in particular its volume adequacy indicator, its overall ease of

use and comfort, also the handle of the collector was too rigid and the absorbent material was too hard.

Method of collection	Advantages	Disadvantages
OMT collection	Ease of collection No discomfort felt Could not feel insertion of filter paper strip	Requires a dentist
Drooled Saliva	Ease of collection	Aversion to spitting Being able to see collected saliva Unpleasant
Orasure	Ease of collection Comfortable in mouth Self directed	'Salty' after taste in mouth
Salivette	Easily positioned in oral cavity (Superior/ Inferior to tongue)	After taste was unpleasant. Awkward feeling when placed in mouth.
Oracol	Ease of use Very comfortable	Slight After Taste
Seradate	Comfortable around the gums	Too large Pads fall into mouth Slight discomfort
Certus	Highly aesthetic design (volume adequacy indicator was commended) Ease of use Comfortable	Handle is too rigid Pad material is too hard

Table 2.2 The Advantages and disadvantages of methods of collection

2.6 Discussion

The development of the Seradate

The early stage in the development of Seradate was to create a collector that resembled an OFCD currently available on the market. The design of a toothbrush and the method of collection were taken from that used in an oral swab. This would allow for a sample to be collected that would show OMT-rich oral fluid collection. This method is well established as a toothbrush and an OFCD. The marketability for such a device would be poor, as many commercial collectors boast the same function; this would not be a valuable addition to the oral fluid collector market. What was required was a unique method of oral fluid collection that will use established collection methods as a basis and to improve the collection of OMT-rich oral fluid.

The stage 2 design was based on the findings stated by Crouch et al that sample collection itself requires standardisation and the levels of oral fluid collected can affect the test outcomes from a variety of validation tests (Crouch 2005). This led to suggest that the ability to collect a greater volume of oral fluid would allow the one sample collection to provide enough fluid for multiple tests and further validation applications. The stage 2 designs used this information taking into account a currently used oral interface (the mouth guard), and this was an ideal concept as it provided all the advantages that were required to collect a sample that was OMT-rich. The guarding elements of the design would be used to shield the gums from the external oral cavity and any salivary interaction, making the collector site specific for OMT collection. The design initially targeted both the upper and lower portions of the gums to

efficiently collect two samples at the same time; this was to lower overall screening time that may require multiple collections.

The design itself had its limitations as both the upper and lower gums were targeted for interaction; this limited the biting mechanism that could be used to increase OMT outflow. The stage 3 designs collectively assessed the findings from stages 1 and 2 and merged the 2 concepts to form an OMT mediated oral fluid collector. The idea of using the toothbrush handle was removed due to the unique mechanism of collection used. The targeting of the lower gums allowed for the device to be easily placed within the oral cavity and the additional curved coronal region provided an easy biting point.

The prototype incorporated two forms of collection into its test design this included the abrasion motion and the biting motion. The dual functions of the Seradate were proposed to facilitate an increase in OMT outflow by causing mild trauma to the surfaces of the gingivae.

Seradate absorbent materials

The materials used for the Seradate collection head were tested for their hydrophilic properties, ability to collect and release water/ oral fluids while exhibiting minimal retention and overall comfort for placement within the oral cavity.

Each of the tested materials showed a high affinity for both oral fluid and distilled water, this was the first experiment conducted to measure the speed of complete saturation (data not shown).

The tested materials were a mixture of polyolefin and sintered plastics. Each material was individually assessed for its acceptability (Table 2.1).From our findings the shortlisted materials for use in the Seradate were BNW-1 (polyolefin), D3727g (polyolefin) and XS-2 (sintered plastic). Each material met the requirements for use within the Seradate but as D3727g exhibited a greater volume uptake of H_20 and oral fluid in comparison to BNW-1 and XS-2 this material was selected for the Seradate.

D3727g showed the appropriate properties that were suited for our collector. The material could be easily molded and was comfortable enough to be placed within the oral cavity. It also showed a high uptake and release of tested fluids, with a low retention of testable proteins (IgG).

XS-2 was a sintered plastic and as the Seradate used both abrasion and mastication as a method of collection this would cause excessive trauma to the gum surfaces (resulting in bleeding) and therefore nullify its application as an appropriate OFCD. The potential uses for XS-2 would be as a complimentary collector that is placed on the gum surface to allow transfer of fluid by capillary action.

Site specific applications of wicking based materials such as sintered plastics require to be assessed further for their appropriate use in intra oral dental examinations.

User compliance questionnaire

To measure user compliance of our tested OFCDs, we looked at the following parameters

- Aesthetics
- Ease of use
- Comfort
- Overall Experience

The results shown previously were taken from a cohort of male and female participants.

Aesthetics

Aesthetics was used a measure of first impressions on its visual aspects and not on its direct application. Our findings showed that each collection method was deemed acceptable against our cut-off. There was no significant difference between the Orasure, Salivette, Seradate and Certus in comparison to the control (drooled saliva). Oracol showed a significantly higher value in comparison to OFCDs, OMT collection was significantly higher in comparison to all collection methods.

Drooled saliva collection was highlighted as the least aesthetic as participants were able to see their own drooled saliva. This could be averted in future collections by having the participants drool into opaque containers. The Oracol was commended on its simplistic design (like a lolly pop).

The design of the Certus was commented on as having the best look in particular the window for the adequacy indicator and the overall design of the construct was highlighted.

Ease of use

Ease of use was to check the simplicity of each collection procedure after being given both written and verbal instruction.

The collection methods were assessed for their ease of use and each method was deemed acceptable by our cohort. There was a universal acceptance for the collection of OMT (both intra and extra-crevicular collection) conducted by the dentist. There was no significant difference between the Orasure, Salivette, Seradate and Certus in comparison to the control (drooled saliva). Oracol showed a significantly higher value in comparison to OFCDs, OMT collection was significantly higher in comparison to all collection methods. Oracol was commended on its overall ease of use.

Comfort

The comfort level for each collection method was a measure of any pain or discomfort derived from each of the collection procedures. Each of the collection methods was deemed acceptable as they fell above our cut off. There was no significant difference between the Orasure, Salivette, Seradate and Certus in comparison to the control (drooled saliva). Oracol showed a significantly higher value in comparison to OFCDs, OMT collection was significantly higher in comparison to all collection methods.

OMT collection was commended on the feeling of no discomfort, each of the collection methods were commended on particular aspects of comfort. The Seradate was commended on being comfortable around the gums, however the size of head was commented on being too large and the inserted absorbent material was too large causing it to fall out of the applicator. The Oracol was commended for its pleasant material texture.

Overall experience

The overall experience was used as universal measure to encompass all previous aspects of the questionnaire and whether the participants were willing to do a repeat procedure if the need arose. Each of the collection methods was deemed acceptable as they fell above our cut off (5 and above). There was no significant difference between the Orasure, Salivette, Seradate and Certus in comparison to the control (drooled saliva). Oracol showed a significantly higher value in comparison to OFCDs, OMT collection was significantly higher in comparison to all collection methods.

The collection of OMT was unanimously agreed on as the best overall experience, the cohort agreed that the procedure was very simple and pain free. The experience of drooling saliva was least liked and was commented on as being unpleasant. The Orasure, Oracol and Salivette were commended on collection method however each of the collectors showed a distinct after taste that was mildly unpleasant. All participants did comment that regardless of this factor they would be happy to use the collector again in the future. The Certus was credited for its overall design as an applicator and especially its volume adequacy indicator; its drawbacks were highlighted in terms of its rigidity of the absorbent material. The Seradate was commended on its material use and comfort upon placement in the mouth in particular the gum surfaces. Contraindications were that the head of the applicator was too large and a reduction in the size of the absorbent material would have overall enhanced the experience.

Closing comments

As the design of the Seradate was a working prototype it still has a lot of scope for development and taking into account the constructive criticism it could be used as a potentially marketed OFCD in the future.

Chapter 3

An evaluation of oral fluid collection devices and their use in point of care diagnostics

3.1 Introduction

The collection of oral fluid is an attractive alternative matrix to serum for use in medical diagnostics and screening. There are many methods by which oral fluid is collected and for the purposes of this thesis we want to evaluate a group of OFCDs against Seradate our prototype OFCD. Each of the OFCDs will be evaluated for its ability to monitor Immunoglobulins IgA, IgG, IgM and cotinine.

OFCDs and immunoglobulins

The purpose of collecting the immunoglobulins in oral fluid are its uses as a surrogate marker for OMT are well established within the literature (Malamud 1997). Dimeric Salivary- IgA (S-IgA) is predominantly produced by the parotid and submandibular glands and is found in higher titre in oral fluid, the concentration of IgG and IgM are considerably lower in comparison; this is due to their presence being associated to OMT outflow. The collected levels of IgG and IgM are often masked in oral fluid samples, due to dilution. (de Almeida et al 2008, Childers et al 2003). A ratio of IgG: IgA levels is a useful indicator to mark the presence of OMT to oral fluid based immunoglobulins when they are presented in collected oral fluid.

OFCDs and monitoring disease

OFCDs have been used to diagnose various blood borne diseases and vaccination status these include HIV/Hepatitis B and C, the presence of viral infection can be detected by antibodies against the virus and their presence in collected samples provide an adequate monitoring method that can be comparable to values monitored in serum. (Nokes et al 1998, Nurkka et al 2003, Vyse et al 2001, Yacoubian et al 2006).

OFCDs and monitoring drugs/hormones

OFCDs are also used to monitor levels of therapeutic drugs, illicit drugs, hormone monitoring and provide concentration levels of unconjugated free-drugs and hormones that are present in blood circulation (Malamud 1997) (Cameron and Carman 2005) (Vyse, Cohen et al. 2001; Shirtcliff and Marrocco 2003)

The monitoring of therapeutic and illicit drugs has been associated with oral fluid testing with a wide range of analytical methods currently available. The methods of analysis include initial quantitative screening by immunoassay (ELISA) and confirmatory analysis is conducted by mass spectrometry or western blotting. This is particularly valuable in low compliance groups which include the elderly, young children, haemophiliacs and venous compromised individuals

One use of drug monitoring in oral fluid has been to evaluate smoking status and daily smoking habits. This can be done by measuring cotinine levels (a breakdown product of nicotine). Smoking has been linked to various diseases and government initiatives to promote smoking cessation; cotinine testing is employed as a deterrent for smoking relapse. Smoking and

disease are inter-related the establishment of smoking status is of great use for clinical assessment. With the use of non-invasive OFCD screening methods it is relatively simple to measure smoking more accurately than by individual admission. As smoking is dictated by levels of nicotine presence this has a relative short half-life of 2-4 hours. Its main metabolites are cotinine and Nicotine N-Oxide. Cotinine is a lot more stable with a half-life of around 16-24 hours in vivo (Jacob Iii, Yu et al. 2011) its approximate levels of collection can be measured via immunoassay and secondary confirmation of cotinine can be conducted using Liquid Chromatography (LC) or Gas Chromatography-Mass Spectrometry (GC-MS).

Factors affecting OFCD collection

The volume of oral fluid collected is of importance when related to the availability of adequate samples for testing. The volume of fluid collected has a direct consequence on the dilution of compounds present within the fluid. As many OFCDs claim adequate volumes of collection, the Concateno Certus collector has introduced a method of standardising the collected sample by inserting a volume adequacy indicator (approximately 1 ml). The limitations of unknown volume collection affect the final concentration of compounds within an oral fluid sample; this sample is diluted further in OFCDs that use a preservative solution. The assumption of sample volume will affect the final concentration values of the collected test analytes. The possibility of retention of oral proteins within the absorbent material should also be taken into consideration (Holm-Hansen 2004,Crouch 2005).

The onset of clinical implications such as Xerostomia (dry mouth) can affect the levels of oral fluid collected; this is associated to

anxiety prior to sample collection or from dehydration. Current methods stipulate the use of the OFCD for a period of 2-5 minutes to amend this, but this doesn't take into account physiological and mental effects induced by the collection procedure (Aps et al 2005, Drummer 2008).

The diurnal nature of oral fluid and inter and intra variability seen in collection doesn't allow for standardisation of proteins available in oral fluid. Precautions should therefore be taken when comparing data taken at different time sets.

3.2 Aims

Our aims are to compare the Seradate against various marketed OFCDs which include the Orasure, Salivette, Oracol and Concateno Certus. As our interest in oral fluid resides on the collection of OMT based constituents, we will look at each of the OFCDs ability to release IgG (our surrogate marker for OMT) from a pooled sample of known concentration of IgG. As oral fluid Immunoglobulin levels are variable, a set of standards will be used to measure IgG levels of pure OMT (GCF) against OFCDs with drooled unstimulated oral fluid as control. Pure OMT will be collected using the filter paper strips the methods of collection used will be intra-crevicular and extra-crevicular.

As the samples will be collected in the same time period, an assessment in sample collection will also be conducted to account for variation in levels of IgG and cotinine measured. This will help establish whether oral stimulation can return to normal with a short resting period after each sample is collected.

The ability of the Seradate and OFCDs to release oral fluid compounds will be measured by monitoring the collection and release of IgG/IgM/IgA and cotinine. As OFCDs and drooled oral fluid samples, show a mixture of antibody content, the IgG: IgA ratio will also be measured. The order of collection of samples will also be tested to assess for bias within the study. The study was conducted on group of student volunteers aged between 18-30, composed of a mixture of male and female students that are smokers and non-smokers (ethical permission was granted by the University of Liverpool ethics committee see appendix A)

3.3 Materials & Methods

A comparison in volume of oral fluid collected by OFCDs

The volume of collected oral fluid by an OFCD is a required assessment for availability of sample for testing procedures as described by Crouch et al (Crouch 2005). The volume of oral fluid collected was measured by placing each OFCD in a pooled sample of Oral Fluid (10ml) for a period of 2 minutes or until fully saturated. Before centrifugation, the volume of the released fluid from the device was measured using the following method

Total volume of oral fluid collected (ml) = Saturated weight of OFCD (g) – Dry weight of OFCD (g)

The volume of oral fluid retained by OFCDs

The volume of Oral fluid retained within each collector after centrifugation, is an indication of the level of sample that is not available for testing. To measure the retention of oral fluid in the OFCDs, the collectors were centrifuged and weighed (final weight). The following equation was used

$$\text{The percentage of Oral fluid retained (\%)} = \frac{\text{Final Weight (mg)}}{\text{Dry Weight (mg)}} * 100$$

Assessing the retention of IgG/IgM/IgA in OFCDs

Measuring immunoglobulin concentration

Human IgG/IgM and IgA quantitation kit (Bethyl Laboratories, Montogomery, USA) were used according to manufacturer's instructions. 96 well High Bind flat bottomed plate (Costar Corning Lifesciences, UK) were coated with goat anti-human affinity antibody (Primary Antibody, Bethyl Laboratories Inc, USA) .The number of wells used were determined by assay standards, controls and the total number of samples tested (either duplicate or triplicate). 1 µl of capture antibody was diluted with 100 µl of coating buffer (0.05 M Carbonate-Bicarbonate Sigma Aldrich, UK) administered to each test well. The plate was incubated at 21°C for 60 minutes or stored overnight at 4°C.

The coating buffer was removed by a 3 - step wash using a Tecan Columbus plate washer (Austria) with PBS 0.05% Tween 20. Each step of the wash involved washing of the wells and aspirating the wells, this process was repeated a total of three times within the 2-step wash process, the plates were dried by tapping onto blue roll. 200 µl of blocking solution (Phosphate buffered saline, 1% BSA, pH 8.0) was added to each well. The plates were incubated for 60 minutes at 37°C. After incubation the blocking solution was removed by 3 step wash (as previously described).

A set of assay standards were made using human serum as reference (final serum concentration 1000 ng/ml according to manufacturer's guidelines). 100 µl of sample diluent (Phosphate buffered saline 1% BSA, 0.05% Tween 20, pH 8.0) was added to each well. 100 µl of dilute standard was added to the first well, mixed and 100 µl removed and added to the second well, the process was repeated for the next 7 wells. This produced a standard curve ranging from 1000 ng/ml - 7.8 ng/ml. Oral fluid

samples were then added these were diluted to optimal concentration (1:100 dilution from neat) to specific wells. The plates were then incubated at 21°C for 60 minutes. The samples were removed by 5 step wash (This is a similar process to the 3 step wash). Secondary HRP detection antibody was added to each test well, this was diluted from stock (1:50,000).100 µl of secondary HRP-detection antibody was added to each well and the plate incubated for 60 minutes. After Incubation the secondary HRP-detection antibodies were removed using a 5-step wash and 100 µl 3, 3', 5, 5'-Tetramethylbenzidine (TMB. Sigma Aldrich) was added to each well. The plate was sealed, covered and placed on a rotating platform for colour development. The development time was between 5-10 minutes, the reaction was stopped by adding 100 µl 4N H_2SO_4 to each test well. The plate was analysed at 450 nm on a spectrophotometer (J Bio LP 400). The sample Immunoglobulin concentrations were calculated from a standard curve using Microsoft Excel (For a detailed list of materials used please refer to appendix B).

Assessing the retention of IgG in OFCDs

A comparison between OFCDs was made using oral fluid that was spiked with a known concentration of IgG (60 ng/ml). Each collector was tested in 5 repetitions to account for any differences in IgG retained. Each device was immersed within the pooled sample for 2 minutes before being centrifuged. The amount of IgG retained by each device was calculated by subtracting the final concentration measured from the initial pooled concentration. The Value of retention was then calculated as a percentage.

The percentage of IgG retention

= Original pooled sample/Final Collected sample * 100

Collection procedures for OFCDs and OMT

Unstimulated oral fluid

Volunteers were asked to drink and swill water in their mouths prior to drooling. The volunteers would then allow saliva to drip from lower lip with no additional stimulation into a 25ml universal tube (Sarstedt, Germany). The collected saliva varied between 1.5 ml - 4 ml. Samples were aliquot into 1.5 ml microfuge tubes (Eppendorf, UK) and centrifuged at 8000g for 5 minutes to remove any cellular or particulate matter. The supernatant was removed and 2 µl of protease inhibitor was added to each sample. The samples were stored at -80°C until required.

Seradate

Volunteers used the Seradate collection device (University of Liverpool, UK) and placed the head of the device just over the molars and premolars of the lower jaw. The Seradate uses two methods of swabbing

(a) Biting down on the head (mastication motion).
(b) Rubbing the device systematically along the lower gum line (agitation motion).

The procedure ranged from 2-5 minutes. The Saturated pad was removed and placed into a 15 ml modified centrifuge tube

(Sarstedt, Germany). The pad was centrifuged at 7500g for 10 minutes. The supernatant was placed into 1.5 ml microfuge tubes (Eppendorf, UK) and 2 µl of protease inhibitor (Roche complete protease inhibitor) was added to each sample. The sample was stored at -80°C until required for testing.

Orasure oral fluid collection device

Volunteers used Orasure collection device (Orasure Technologies Inc. Bethlehem.USA) and swabbed around the cheeks and gums, leaving it to rest in the upper right cheek for a period of 2 minutes. The swab was transferred to a preservative vial. The swab centrifuged at 7500g for 10 minutes. The contents were aliquoted into 1.5 ml microfuge tubes (Eppendorf, UK). The samples were stored at -80°C until required for testing.

Salivette

Volunteers used Salivette collection device (Sarstedt, Germany) and chew the swab for a period of 2 minutes. The swab was removed and placed into its housing tube. The swab was centrifuged at 7500g for 10 minutes. The supernatant was collected and aliquoted into 1.5 ml microfuge tubes (Eppendorf, UK) and 2 µl of protease inhibitor (Roche complete protease inhibitor, Roche, UK) was added to each sample. The samples were stored at -80°C until required for testing.

Oracol

Volunteers used the Oracol collection device (Malvern Medical Developments, Worcester, UK) and swabbed around the cheeks and gums, leaving it to rest in the upper right cheek for a period of 2 minutes. The swab was transferred to a 15 ml centrifuge tube (Sarstedt, Germany). The swab was centrifuged at 7500g for 10 minutes. The supernatant was aliquoted into 1.5 ml microfuge tubes (Eppendorf, UK). 2 µl of protease inhibitor (Roche complete protease inhibitor, Roche, UK) was added to each sample. The samples were stored at -80°C until required for testing.

Concateno Certus

Volunteers used the Concateno Certus collection device (Concateno global drug testing services, London UK) and swabbed around the cheeks and gums, leaving it to rest in the upper right cheek for a period of 2 minutes. The Swab was removed once the volume adequacy indictor turned blue. The swab was transferred into a tube containing preservative and centrifuged at 7500g for 10 minutes. The content was aliquoted into 1.5 ml microfuge tubes (Eppendorf, UK). The samples stored at -80°C until required for testing.

Pure OMT (GCF) collection

Intra-crevicular OMT collection

Volunteers were asked to rinse their mouths with water. The procedure was carried out by Dr Bhavish Patel BDS (VU Manc) (GDC Number 74574). Dental rolls were positioned under the superior lip to dry area of collection. The area of collection was the upper central incisor and upper lateral incisor. The area was cleaned with gauze to remove any food matter or supraginvival plaque. Filter paper strips (1 cm * 3cm) was placed intra-crevicularly into the gingival crevice until mild resistance was felt (figure 3.1). The filter paper was then left in place for a period of 1 minute and removed.

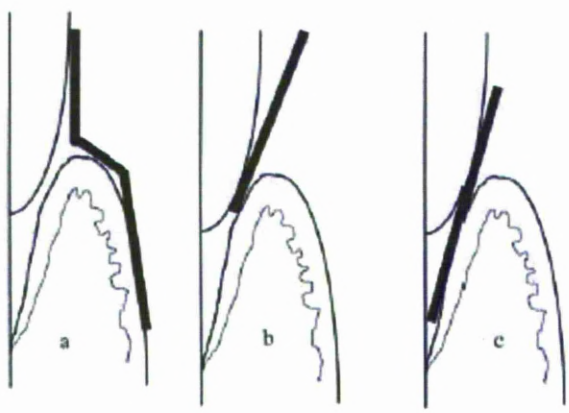


Figure 3.1 An illustration of the positioning of paper for the filter paper strip method of collection (a) Extra crevicular (b) Intra crevicular- Superficial (c) Intra crevicular- Deep. Modified from Griffiths GS 2000.

To measure the volume collected the filter strip was placed into a microfuge tube and weighed. The filter paper after collection was placed into the microfuge tube and re-weighed. The sample

volume collected was worked out by subtraction of final weight from initial weight. 300 µl of PBS Tween 0.05% was added to each strip and the sample was stored at -86°C. For processing the samples were thawed and centrifuged at 7500g for 10 minutes and agitated on a roller mixer for 20 minutes. The filter paper was then removed and the sample was used for testing.

Extra-crevicular OMT collection

The collection procedure followed similar methodology as Intra-crevicular collection. The area of collection was the upper central incisor and upper lateral incisor. Filter paper strips (1 cm * 3cm) were placed at top of the gingival crevice. The filter paper was left in place for a period of 1 minute and removed (see figure 3.1).
To measure the volume collected the filter strip was placed into a microfuge tube and weighed. The filter paper after collection was placed into the microfuge tube and re-weighed. The sample volume collected was worked out by subtraction of final weight from initial weight. 300 µl of PBS Tween 0.05% was added to each strip and the sample was stored at -86°C. For processing the samples were thawed and centrifuged at 7500g for 10 minutes and agitated on a roller mixer for 20 minutes. The filter paper was then removed and the sample was used for testing.

Standardisation of IgG collection for OFCD

To standardise our testing of OFCDs, the same volunteer group were used from the OMT collection study. The cohort comprised of male and female, smokers and non-smokers. The order of sample collection was carried out in a manner to reduce agitation of the gum surfaces to lower false results. Each procedure was followed by a resting period (approximately 15 minutes) to allow for oral fluid composition to re-establish itself.

Measuring cotinine levels in oral fluid

Oral fluid samples collected were assayed for cotinine levels using High Sensitivity salivary cotinine assay kit (Salimetrics Europe Ltd). All reagents and plates were brought to room temperature. 1X wash buffer was prepared. Serial dilutions of Cotinine standard were made in microfuge tubes to create a standard curve ranging from 200 ng/ml - 0.8 ng/ml. The Samples were diluted 1:10 in sample diluent for smokers and left undiluted for non-smokers. 20 µl of standards, control and sample was placed into an appropriate well. 20µl of assay diluents was placed into 2 wells to serve as control. Each sample was run in duplicate. The enzyme conjugate was diluted 1:300 (with assay diluent). 100 µl of anti-serum was added into all test wells using a multichannel pipette. The plate was covered and placed in an incubator/shaker for 90 minutes at 37°C.

The test samples underwent a four-step wash 4 (using Tecan Columbus Plate Washer, Austria). The plate was Blot dried and 200 µl of 3, 3'5, 5' - Tetramethylbenzidine solution into each well. The plate was mixed at 500 rpm for 5 minutes and incubated in dark for 25 minutes at room temperature (21°C). 50 µl of stop solution was added to each test well and the plate was analysed at

450 nm on a spectrophotometer (J Bio LP 400). The cotinine concentrations of the samples were worked from a standard curve using Microsoft Excel.(For a detailed list of materials used please refer to appendix B).

OFCDs and monitoring of IgG and cotinine

To assess bias within our order of sample collection, the samples were taken on 3 separate occasions. The collection order was as follows

- Normal collection order (Saliva, Orasure, Salivette, Oracol, Seradate, Certus)
- Reverse order* (Certus, Seradate, Oracol, Salivette, Orasure, Saliva)

*= The reverse order was collected twice

3.4 An assessment of Seradate and OFCDs

Comparing OFCD oral fluid and IgG retention

Each OFCD was tested in 5 repetitions to account for any differences in volume retained (Figure 3.2). The Seradate shows a significantly higher level of oral fluid collection in comparison to the Orasure, Salivette, Oracol and Certus with an approximate uptake of 4 ml. The Salivette shows a significantly higher collection of oral fluid in comparison to the Orasure, Oracol and Certus (≈3.5 ml. fluid uptake).The Orasure and Certus show comparable levels of oral fluid collection at approximately 1 ml. This value is significantly lower in comparison to the Salivette and Seradate, and only marginally higher in comparison to Oracol. The Oracol showed least volume collected amongst all tested OFCDs at approximately 0.8 ml and was significantly lower in comparison to the Orasure, Salivette, Seradate and Certus.

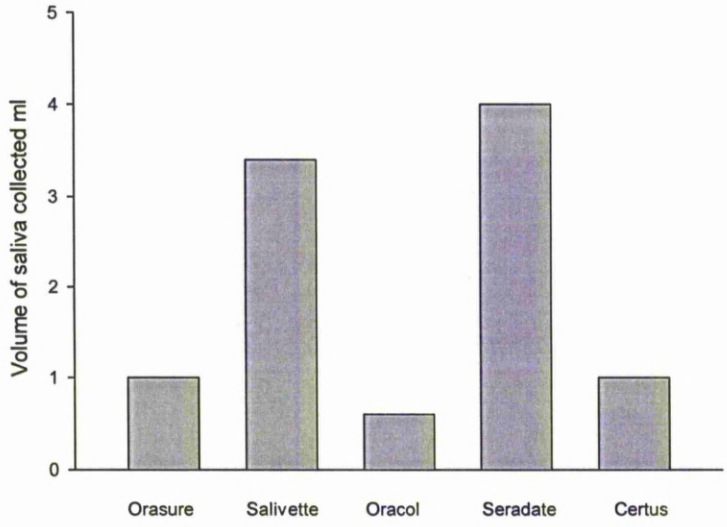


Figure 3.2 The volume of oral fluid collected after centrifugation from tested OFCDs. There are no error bars because the deviation from the mean was too small. n=5

The volume of oral fluid retained by OFCDs

Each collector was tested in 5 repetitions to account for any differences in percentage of volume retained within each OFCD (see figure 3.3).The Concateno Certus was excluded from this test as its methodology doesn't use centrifugation to release fluid. The Salivette and Oracol retained approximately 17% of total oral fluid this was significantly higher in comparison to the Orasure and Seradate. Orasure retained approximately 10% oral fluid; this was significantly lower in comparison to the Salivette and Oracol, but only marginally higher in comparison to the Seradate. The Seradate retained approximately 2% of the oral fluid and was significantly lower in comparison to Orasure, Salivette and Oracol. The retention of oral fluid within the tested OFCDs can provide answers to final concentrations of proteins presented for testing; this can either show an increase due to greater retention of oral fluid captured within the material or be caused by entrapping proteins within the material.

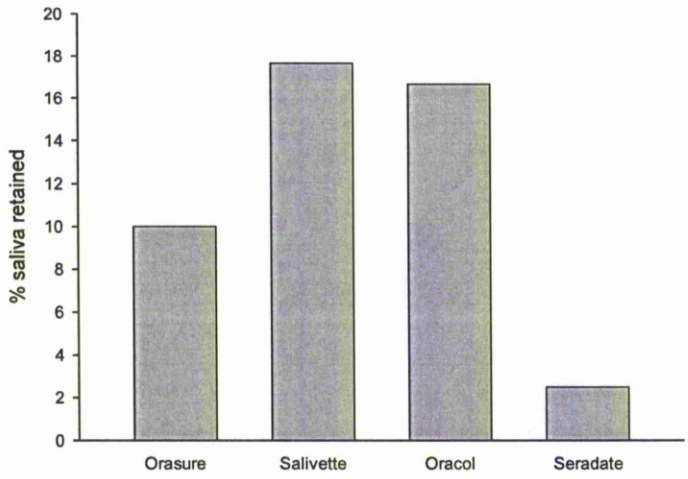


Figure 3.3 The percentage of oral fluid retained in OFCDs. There are no error bars because the deviation from the mean was too small. n=5

Assessing the retention of IgG in OFCDs

Each collector was tested in 5 repetitions to account for any differences in percentage of IgG retained within each OFCD (see figure 3.4). The Certus retained approximately 55% of the total IgG and was significantly higher in comparison to the Orasure, Salivette, Oracol and Seradate. The Orasure retained approximately 25% of total IgG and was significantly higher in comparison to Salivette, Oracol and Seradate. The Seradate retained approximately 10% IgG and was significantly higher in comparison to Oracol, But significantly lower in comparison to Orasure, Salivette and Certus. The Oracol showed least retention of IgG at 5% and was significantly lower in comparison to all tested OFCDs. The retention of un-testable IgG in each collector accounts for 15-30%. The retention of IgG is one parameter that can cause invalidation of an oral fluid sample, should the collected sample have a low IgG level below cut-off values used in oral fluid sample validation.

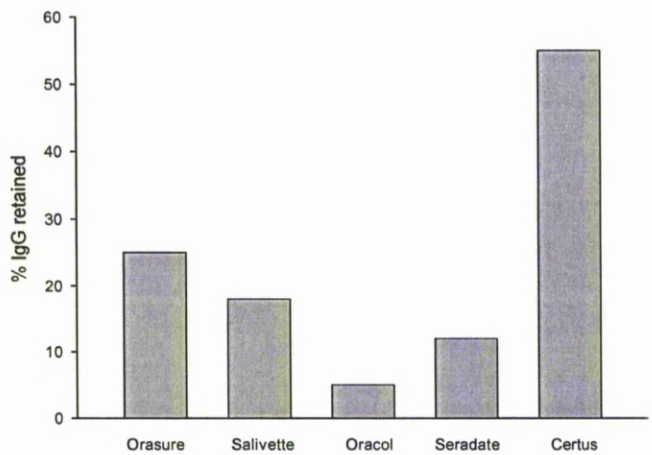


Figure 3.4 The percentage of IgG (60ngml) retained from spiked oral fluid samples. There are no error bars because the deviation from the mean was too small. n=5

3.5 Standardisation of IgG collected in oral fluid

A comparison of different OMT collection methods

The collection methods were evaluated by measuring IgG levels (figures 3.5 and 3.6). The test cohort was separated into 2 groups male and female and also smokers and non-smokers to show any differences in OMT collected. Using the intracrevicular method the male volunteers gave values marginally higher to the female cohort approximately 150 µg/ml in comparison to 125 µg/ml. With the intracrevicular method there was no difference between the two groups (≈80 µg/ml). There were no overall significant differences between the two groups.

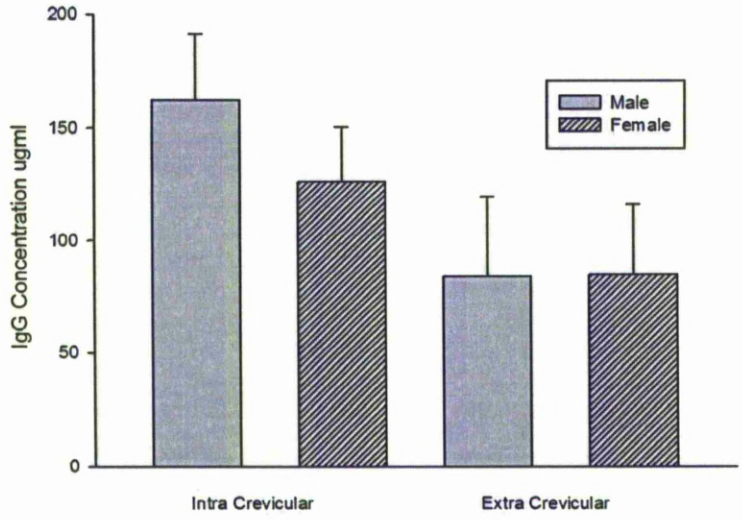


Figure 3.5 IgG concentration of pure OMT (GCF) via intra-crevicular and extra-crevicular collection methods. Bar show differences between male and female samples. Bar height represents the mean value ± SE. n = 20

Within the smoking cohort, the intra-crevicular collection was marginally higher in comparison to the non-smoking cohort approximately 130 µg/ml in comparison to 100ug/ml. For the extra-crevicular collection the similar trend was witnessed with the smokers exhibiting a marginally higher amount of IgG approximately 80 µg/ml to 60 µg/ml. There were no overall significant differences between the two groups. The participants were not subjected to abrasion to gum surfaces and gum health of the individuals was assessed by the dentist. Elevations in OMT IgG levels would have been seen in compromised groups with a range of poor oral health. The two collections showed lower values in comparisons to ranges dictated within the literature.

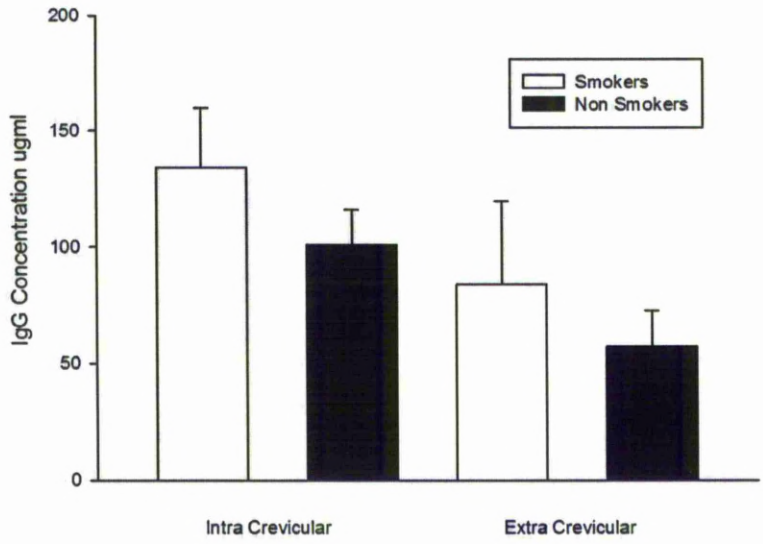


Figure 3.6 IgG Concentration of pure OMT (GCF) via intra-crevicular and extra-crevicular collection methods. Bar show differences between smoker and non smoker samples. Bar height represents the mean value ± SE. n = 20

Standardisation of IgG collection for saliva and OFCD

Comparable values for IgG were seen between the Orasure, Seradate and the control (drooled oral fluid ≈ 4 µg/ml), these values were significantly higher in comparison to the Salivette (≈1 µg/ml) and Certus (≈1 µg/ml), both of which expressed low levels of IgG collection (figure 3.7). The Oracol expressed the highest level of IgG released (≈ 6 µg/ml) and was significantly higher in comparison to all tested OFCDs. The low levels of IgG released by the Salivette are associated to its method of collection, which has lower contact with the gum surfaces.

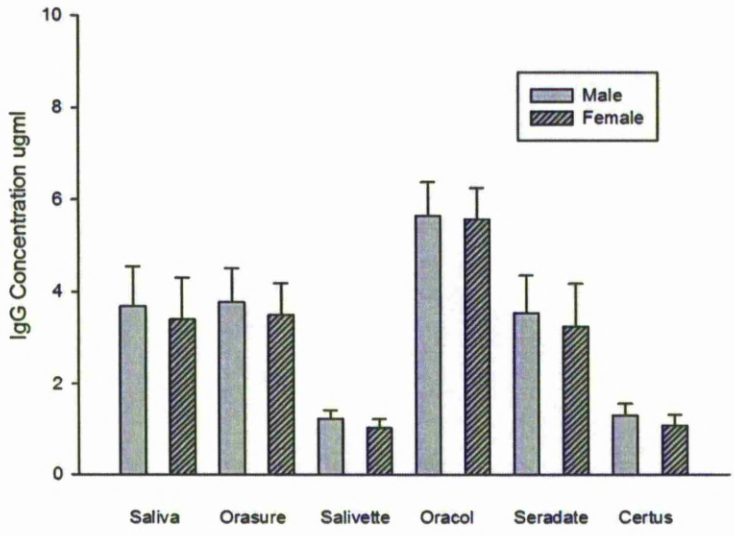


Figure 3.7 IgG concentration of saliva and OFCD columns show differences between male and female samples. Bar height represents the mean value ± SE. n = 20

Similar levels of IgG concentration where seen when comparing the smoking and non-smoking group, with regard to sample collection (figure 3.8). The levels of IgG would be expected to be higher among smokers of poorer gingival health, as our smoking volunteers showed minimal to no signs of inflammation of gingivae, the collections levels between the smokers and non-smokers were comparable

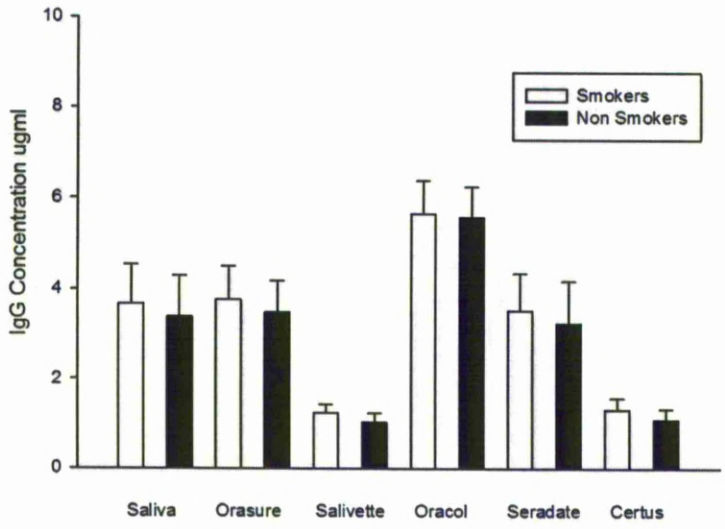


Figure 3.8 IgG concentration of saliva and OFCDs columns show differences between smokers and non-smokers samples. Bar height represents the mean value ± SE. n = 20

3.6 Comparison of immunoglobulins and cotinine present in oral fluid collection

As previous experiments highlighted the differences in IgG released by the OFCDs, we addressed the collection of other immunoglobulins that are present within the oral cavity. The immunoglobulins present in oral fluid are IgG, IgA and IgM.

The examination of Immunoglobulin concentration was used to measure the potential function of each collector in its ability to collect OMT derivatives.

Measuring IgG concentration in oral fluid

The IgG concentration collected by each OFCD was measured against the control (figure 3.9). The levels of IgG collected gave a similar range of values as stated in section 3.4.Comparable levels of IgG concentration were seen between the Orasure, Seradate and control (between 4 µg/ml - 5 µg/ml) this was significantly higher to the Salivette and Certus, and lower in comparison to Oracol. The Salivette IgG concentration (≈ 2µg/ml) was significantly lower in comparison to the Orasure, Seradate, Oracol and control but was significantly higher in comparison to the Certus. The Certus IgG concentration was significantly lower in comparison to all OFCDs (≈ 0.5 µg/ml). The Oracol IgG concentration was significantly higher in comparison to all tested OFCDs (ranging between 5 µg/ml - 7 µg/ml).Within the data it can be seen that the male cohort showed marginally higher levels of IgG concentration in comparison to females.

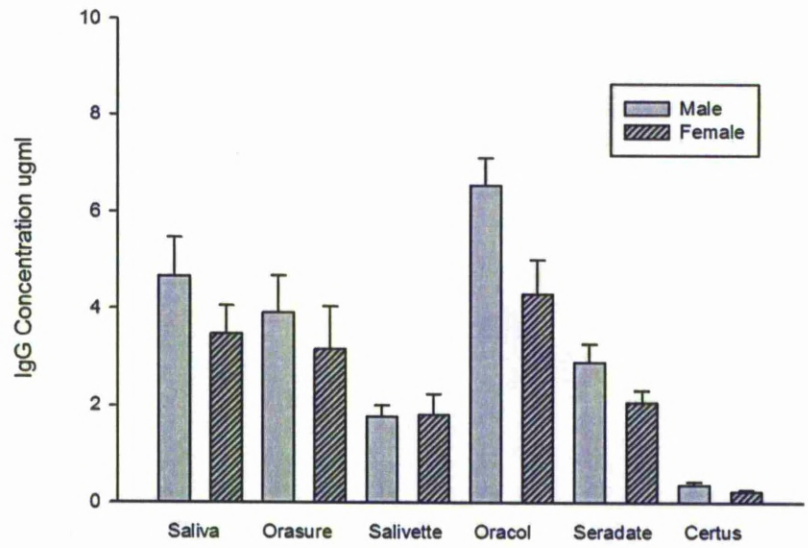


Figure 3.9: IgG concentration of saliva and OFCDs columns show differences between male and female samples. Bar height represents the mean value ± SE. n = 15

Measuring IgA concentration in oral fluid

IgA concentration collected by each OFCD was measured against the control. Comparable levels of IgA were seen between Orasure and Seradate (20 µg/ml - 30 µg/ml), this was marginally lower in comparison to the control (≈35 µg/ml), but significantly higher in comparison to Salivette, Oracol and Certus. Salivette IgA concentration (≈15 µg/ml - 20 µg/ml) was significantly lower in comparison to Orasure, Seradate and control, but higher in comparison to Oracol and Certus (figure 3.10).

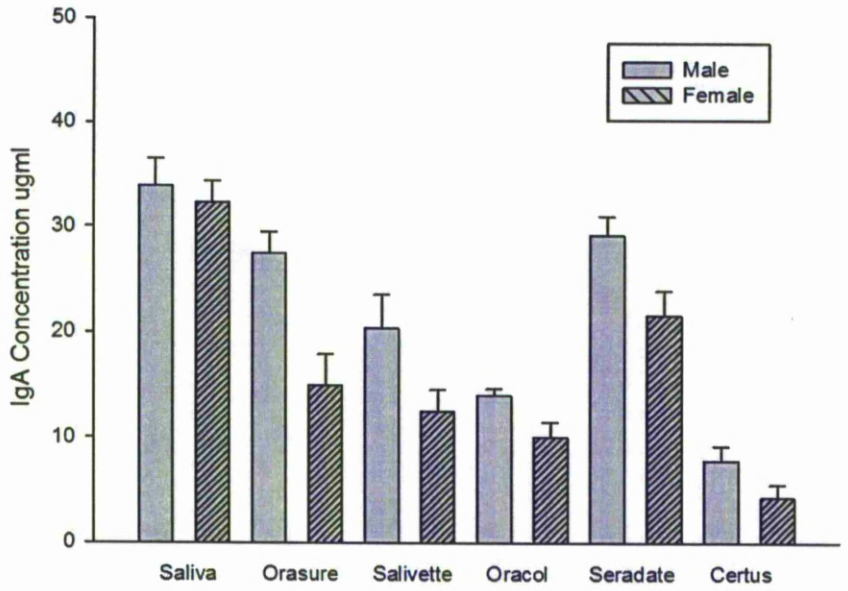


Figure 3.10: IgA concentration of saliva and OFCDs columns show differences between male and female samples. Bar height represents the mean value ± SE. n = 15

Oracol IgA concentration (≈10 µg/ml - 15 µg/ml) was significantly lower in comparison to Orasure, Salivette, Seradate and control. Bur was significantly higher in comparison to the Certus. Certus IgA concentration (≈0.5 µg/ml) was significantly lower in

comparison to all tested OFCDs. The lower values shown in the Oracol can be associated with collection procedure as it is primarily directed to gingival surfaces, and possible retention of IgA within the foam material, this factor can also be witnessed in the Salivette. As the Concateno Certus doesn't utilize centrifugation, this again is a likely limiting factor in its presentation of immunoglobulins.

Measuring IgM concentration in oral fluid

IgM concentration collected by each OFCD was measured against the control (figure 3.11). The male cohort showed marginally higher levels of IgM in comparison to the females. Comparable levels of IgM concentration were seen between the Orasure and Salivette (1 µg/ml - 2 µg/ml) this was significantly lower in comparison to the Oracol, Seradate and control (2 µg/ml - 3 µg/ml), but were significantly higher in comparison to Certus.

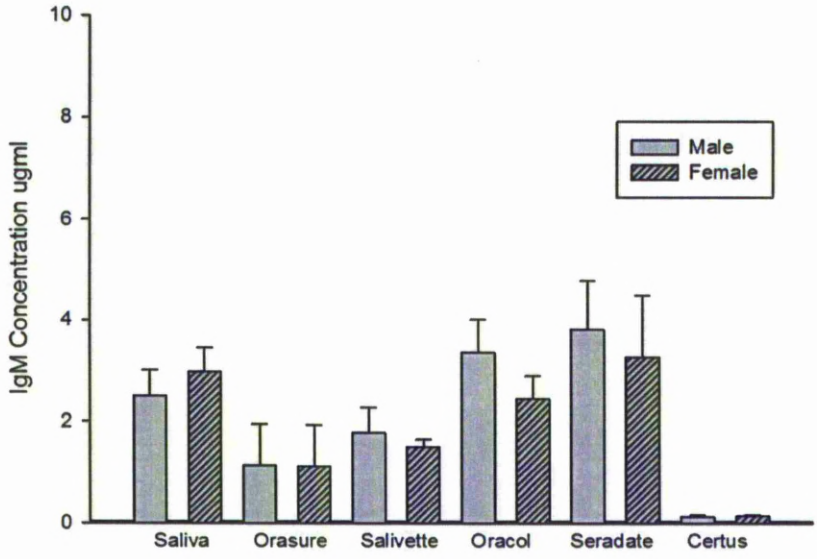


Figure 3.11: IgM concentration of saliva and OFCDs columns show differences between male and female samples. Bar height represents the mean value ± SE. n= 15

Comparable levels of IgM concentration were seen in Oracol and Seradate (3 µg/ml - 4 µg/ml) they were significantly higher in comparison to all tested OFCDs. The Certus IgM concentration (0.2 µg/ml) was the lowest in comparison to all tested OFCDs and control.

As the Certus doesn't utilize centrifugation, this again is a likely limiting factor in its presentation of immunoglobulins.

IgG: IgA ratio

The IgG: IgA ratio can be used as an indicator for affinity for each OFCD in its ability to gain a more OMT-rich sample of Oral fluid. The Oracol showed a higher ratio (0.5) in comparison to all OFCDs (0.1-0.2) and control (figure 3.12). All other tested OFCDs showed similar IgG: IgA ratio that were comparable to the control.

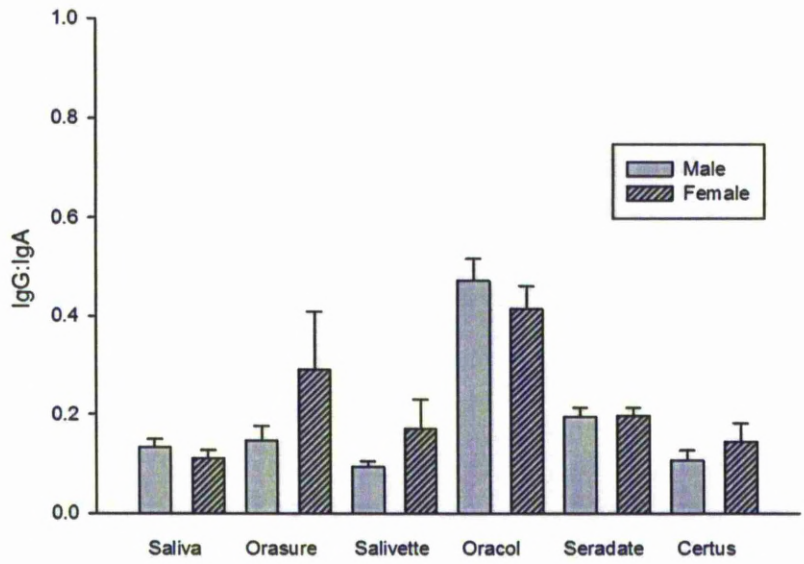


Figure 3.12: IgG: IgA ratio from OFCD and control samples between male and female. Bar height represents the mean value ± SE. n = 15

OFCDS and monitoring of cotinine

The Seradate and OFCDs were tested for their ability to collect cotinine. Cotinine is a derivative on nicotine and is used a primary method of establishing smoking status. Our cohort within the study was a mixed group of males and females with low smoking habit ranging from 2-10 cigarettes per day. Cotinine levels were measured using Salimetrics cotinine assay and it was noted that while the collection procedure would take around 90 minutes to complete, this would have little effect on individual cotinine levels present in vivo. Cotinine half-life within the human body ranges 16-24 hours and is a better indicator than its parent counterpart nicotine which has a shorter half-life of 2-4 hours. Our data in the study showed no significant differences between the collection methods, in comparisons to gender (figure 3.13).

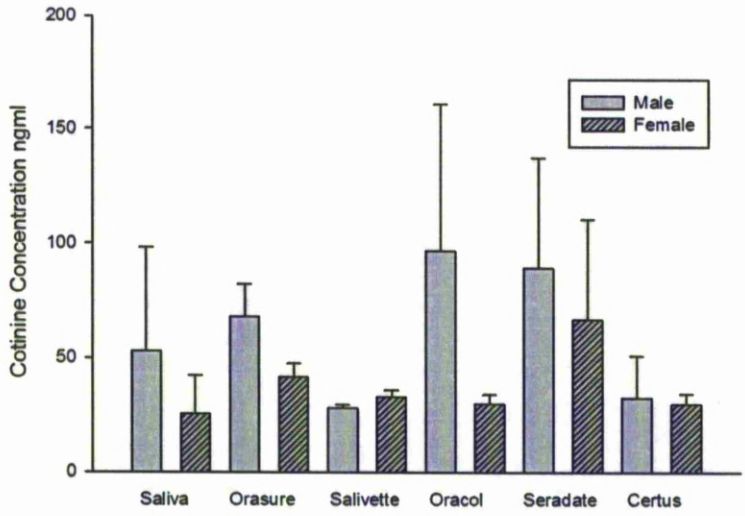


Figure 3.13: Cotinine concentration measured in Saliva and OFCD samples. Columns show differences between male and female samples. Bar height represents the mean value ± SE. n = 20

The range of values of cotinine measured 25 ng/ml -150 ng/ml. The number of male smokers expressed a greater amount of cotinine concentration in comparison to females. The factors are suggestive of males smoking outside of sociable hours or smoking more than their female counterparts. Cotinine charts are suggestive of low to moderate smoking given the values are ranged far below the 300 ng/ml cut-off. There is an elevation of cotinine levels in particular in the Seradate and Oracol OFCD these factors can be attributed to concentration effects of the sample during processing. As both the Seradate and Oracol collection methods samples are collected close to gum line, there is a possibility that they are able to collect a sample of greater concentration.

An assessment of bias in the order of sample collection

OFCDs and monitoring of IgG

In this data we saw differences in IgG levels of the Salivette and Certus (figure 3.14). The Salivette result in reverse collection 1 shows a spurious value (≈ 7 µg/ml), as reverse collection 2 shows a normal IgG concentration. It appears that irrespective of the order of collection the OFCDs all maintain a consistent level of IgG collection. The levels of IgG are comparable with previous collections. The time period of rest between each collection of 15 minutes was sufficient to allow resting periods to return within the oral cavity alongside rinsing of the mouth with water.

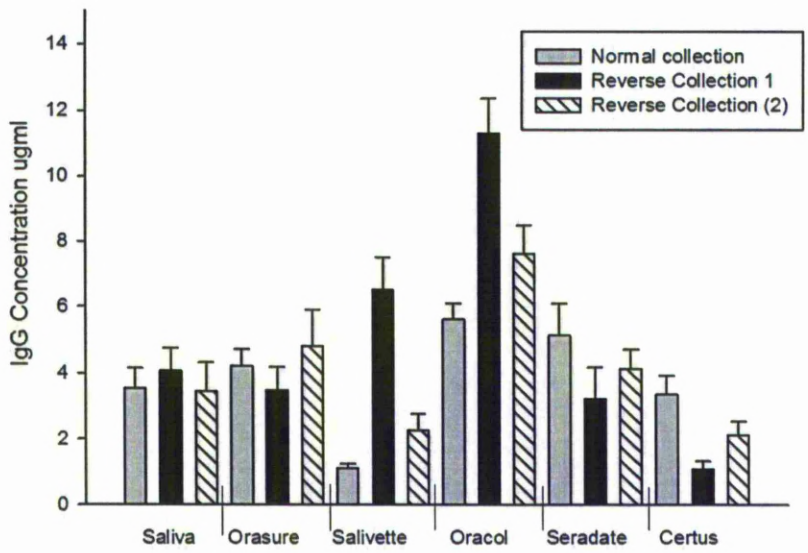


Figure 3.14: IgG concentration of saliva and OFCDs to assess bias in order of collection .Bar show differences between smokers and non-smokers samples. Bar height represents the mean value ± SE. n = 12

OFCDs and the monitoring of cotinine

The collection of cotinine is not as subjective as the collection order bias of IgG (figure 3.15). Cotinine levels will fluctuate with regard to levels of cigarettes smoked, during the separate occasions of collection. The data shows that in each subsequent collection the levels of cotinine are within ranges of the other tested collectors. The bias study shows that cotinine levels are less likely to change either by stimulated or unstimulated oral fluid collection.

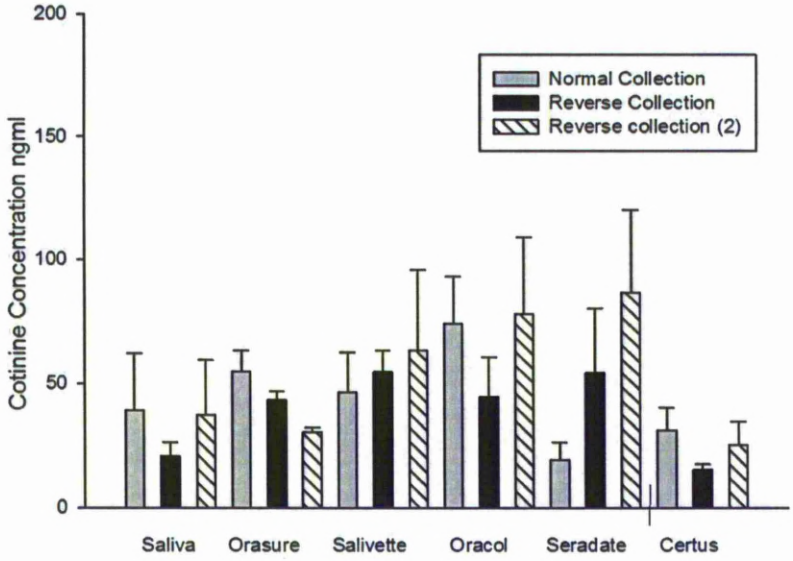


Figure 3.15 Cotinine concentrations of saliva and OFCDs to assess bias in order of collection .Bar show differences between smokers and non-smokers samples. Bar height represents the mean value ± SE. n = 12

3.7 Discussion

The Seradate OFCD prototype and its novel method of use has been placed through a set of stringent tests to provide further information on where the next stage of development in making an OFCD that can collect a higher titre of OMT derived proteins. By creating a set of standards, measurements were taken from a pure source of OMT, OFCDs and normal drooled oral fluid, it's now possible to make comparisons between each method and whether any OFCD truly gains a sample that is close to the values seen in OMT.

With regards to that question the most simple answer is no, a pure sample of OMT as stated in the literature can exhibit values into the region of 200-fold of that seen in our collected oral fluid samples, our collection of OMT has shown levels with that given in the literature. The standardisation of OMT collection is dependent on the method of collection. The collection procedure is non-invasive and final volumes of OMT collected are between 0.2 µl-1.5 µl. The collection of OMT itself is non-standardised and there are many methods of extraction of OMT within literature. Further validation work on the collection of OMT using different filter papers and methods of quantifying the final volume would be useful increasing the accuracy of the data. The sample buffer used in the extraction of OMT further diluted the samples, so it could be used for subsequent testing in assaying for IgG.

As extraction of OMT from filter paper is also subjected to different methods, further sample collections would have helped to elucidate problems faced in quantifying our final volumes. It was also interesting to note that the two methods of extraction of intra-crevicular and extra-crevicular collection didn't differ so greatly. As

the volume of OMT present in such low volume, lower dilution of the strip would've possibly given a higher IgG titre equivalent to that stated in the literature. The levels of OMT are also subject dependant, our group of volunteers all exhibited very good oral health (as assessed by the dentist), and as such the monitoring of immunoglobulins showed comparable data in both male and female volunteers and within smokers and non smokers.

The standards taken from OFCDs showed relatively low significant difference against the control of drooled oral fluid, the values taken by the OFCDs are relative to those listed in previous studies. The exception to the collection was seen by the Oracol OFCD which tested for a greater release of IgG; this factor can be attributed to its foam based material that collected a lower volume of oral fluid in comparison to the other OFCDs. The volume of oral fluid collected has an effect on the final dilution of OMT based proteins; similarities in volume collected were expressed in the Orasure OFCD however its measurement of sample collection is complicated by the addition of its preservative buffer. From our initial findings it can suggested that the material used within the Oracol collector has a greater affinity for OMT capture and release and centrifugation of the sample gives a higher concentration of IgG antibodies. Conversely the Concateno Certus shows a lower level of IgG this value is taken into account of its dilution into its own buffer and the release of oral proteins may not be sufficiently eluted by rinsing of the collector alone. Comparable tests could be conducted on other collectors to see how effectively samples can be eluted in a buffer without the requirement for centrifugation. The idea of not using centrifugation for final processing of the sample would be ideal for less equipped laboratories and provide

greater diversity to the use of oral fluid as a point of care diagnostic tool.

The levels of IgA monitored were significantly higher in the control and Seradate collection device, as our main purpose in the development was to shield from salivary secretions, adequate measures were not in place to account for the collection of saliva from the floor of the mouth where most of the saliva collected, this would account for the higher IgA content witnessed in the Seradate collector. All other OFCDs aside from the Orasure showed lower affinity for IgA collection. As IgA is predominantly released from the salivary glands, it can be suggested that the OFCDS are mainly rubbed on the gum surfaces and there is a possibility of IgA capture within the collectors, this is an additional component that can be validated for future OFCDs in their ability to monitor salivary antibodies.

IgM is generally found in lower volumes in oral fluid due to size of the compound; the levels of IgM were comparable with those collected in IgG but were expressed in a lower level. This suggests that IgM levels are often masked by IgG as an alternate method for validation of an oral fluid sample. With respect to the size of IgM, there is a greater potential to bind to the test material further validation on the release of IgM from respective OFCDs may provide insight into the use of IgM in future studies.

The IgG: IgA ratio was used as an indicator for affinity for each OFCD in its ability to gain a more OMT-rich sample of Oral fluid The Oracol showed highest IgG to IgA ratio, the remaining OFCDs all showed comparable levels. This suggests that Oracol has a greater affinity for IgG in comparison to the other OFCDs and its

selective nature is clearly an attribute that can be further studied in regard to collecting a more OMT-rich sample.

The OFCDs were tested on their ability monitor cotinine the assay used was standardised to account for dilutions in both the Orasure and Certus collectors, the cotinine assay didn't provide much information on our non-smoking cohort as levels for the assay were below the cut-off, the male and females tested for smoking showed varying levels of cotinine in each of the tested collectors in particular the Seradate and Oracol, which showed a slight significant difference in collection in comparison to the OFCDs and control. Possible reasons for the differences are that potentially related to lower sample volume collection or concentration of cotinine within the material.

A collection order of oral fluid samples was studied to see any differences that may have occurred as a result of collecting samples. The samples were collected on 3 separate occasions to account for differences in levels monitored. Initial collection was done in the standard order, and was compared to reverse order of collection on 2 separate occasions. The times of sample collection were kept the same to account for diurnal variability. The final data showed no significant difference in the samples collected. As each method was conducted and allowed for adequate re-establishment of oral cavity at resting state, there was no variation shown.

This was not the case when testing for cotinine in the reverse order, as cotinine levels are dictated by the number of cigarettes smoked, the results showed no significant differences in the collection order, as the levels of cotinine measured almost mirrored the previous data sets.

Closing comments

The testing of the Seradate prototype in comparison to available testing methods has shown that the levels of collection are within the ranges collected by current marketed OFCDs, the collection is a lot lower in comparison to pure OMT collection.

Chapter 4

The effects of temperature on the stability of oral fluid

4.1 Introduction

Oral fluid with its distinct advantages as a non- invasive collection medium can be collected stored and transported at a lower cost in comparison urine and blood, but it suffers from one major factor and that is its' stability after collection. Numerous studies have been conducted on oral fluid sample processing and in particular in the field of oral fluid proteomics (Messana, Cabras et al. 2004; Hu, Xie et al. 2005; Chevalier, Hirtz et al. 2007; Schipper, Loof et al. 2007). Oral fluid is a host to not only endogenous proteases within saliva but also exogenous proteases presented by bacteria within oral flora. Proteases in oral fluid play an important role in the post-translational modification of proline-rich proteins, statherins and cystatins. These proteases regulate breakdown of oral fluid components once saliva is made present within the oral cavity, and the process continues outside of this environment also (Schipper, Loof et al. 2007).

Sample preparation for use in oral fluid diagnostics aims to ensure that constituents of oral fluid remain largely unaltered from time of collection to time of storage. As samples collected for oral diagnosis are often collected on off-site locations from areas of testing, measures are required to be placed that will hinder degradation and to achieve a degree of stability.

Current marketed OFCDs such as the Orasure and Certus take this into account by providing a buffer solution with the collector (Cordeiro, Turpin et al. 1993; Cameron and Carman 2005). The buffer solution is anti-bacterial to lower the effects of degradation

of saliva proteins by endogenous and exogenous factors. The stability tests conducted using the Orasure buffer solution have shown stability of oral fluid test constituents ranging from drugs to immunoglobulins at various temperatures (Malamud 1997; Cameron and Carman 2005).

As most literature based on sample stability covers oral fluid samples collected within a controlled environment, samples are immediately placed on ice for same day testing with the addition of protease inhibitors or subjected to freezing either at -20°C, -86°C or snap-frozen in liquid nitrogen (Cameron and Carman 2005; Hu, Xie et al. 2005; Chevalier, Hirtz et al. 2007). The temperature at which the collected samples can be subjected to prior to analysis is variable on external factors such as location of collection and surrounding temperature.

4.2 Aims

To test the stability of the oral fluid samples and its contents, the concentration of IgG and cotinine were measured. Oral fluid samples were collected using the Seradate and OFCDs (The oral fluid collection and testing of IgG and Cotinine were conducted in the same manner as described in chapter 4).

A cohort of female and male volunteers, were separated into groups of smokers and non-smokers.

Each collected sample was aliquoted into separate microfuge tubes to prevent contamination and to prevent free-thaw errors that can arise in frozen samples. The samples were collected using the Seradate, Orasure, Salivette, Oracol, Certus and normal drooled oral fluid was used as a control.

4.3 Materials and Methods

Stability of oral fluid at different temperatures

To test the stability of the collected samples, each was subjected to a range of temperatures that were documented in the literature. A time course study was conducted with initial values taken on the day of collection and then at intervals of 3, 7, 14, 21, 28 days respectively. The samples were tested for IgG and cotinine concentration using Human IgG Elisa Quantitation Kit Bethyl Laboratories Inc (Montgomery, USA) and Human Salivary cotinine detection kit (Salimetrics Europe, Suffolk, UK).

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Oral fluid storage at room temperature

All test samples were aliquoted, labelled and sealed into 1.5ml microfuge tubes (Eppendorf, UK) and placed on microfuge rack away from direct sunlight and left at normal room temperature. Prior to testing all samples were centrifuged (9000g for 10 minutes) to remove any cellular debris.

Oral fluid storage using refrigeration (4°C)

All test samples were aliquoted, labelled and sealed into 1.5ml microfuge tubes (Eppendorf, UK) prior to placement within a refrigerator. Prior to testing all samples were centrifuged (9000g for 10 minutes) to remove any cellular debris.

Oral fluid storage using freezer (-20°C)

All test samples were labelled and sealed into 1.5ml microfuge tubes (Eppendorf, UK) prior to placement within a freezer. Prior to testing all samples were centrifuged (9000g for 10 minutes) to remove any cellular debris.

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4.4 Monitoring the stability of IgG at different storage temperatures

Stability of IgG at room temperature

Measurements for the stability of IgG from collected samples taken using OFCDs and control at room temperature (See figure 4.1).There was no significant differences in levels of IgG concentration between the male and female cohort.

An elevation in IgG concentration was seen on day 3 post collection in all tested OFCDs. Post day 7 samples that exhibited signs of degradation include Seradate, Oracol, Salivette and Control. These exhibited a similar trend in IgG concentration throughout the time scale. Seradate, Oracol, Salivette and Control were treated with protease inhibitors and showed a significant decline in IgG concentration in comparison to the Orasure and Certus (that used an anti-bacterial buffer).

Post 21 Days the Salivette sample could not be accurately measured as the IgG levels were below the detection range of the assay.

On Day 28 samples collected using the Salivette, Oracol, Seradate and control could not be accurately measured as the Levels of IgG were below the detection range of the assay.

There were no significant changes in the levels of IgG in both the Orasure and Certus over the period of 28 days

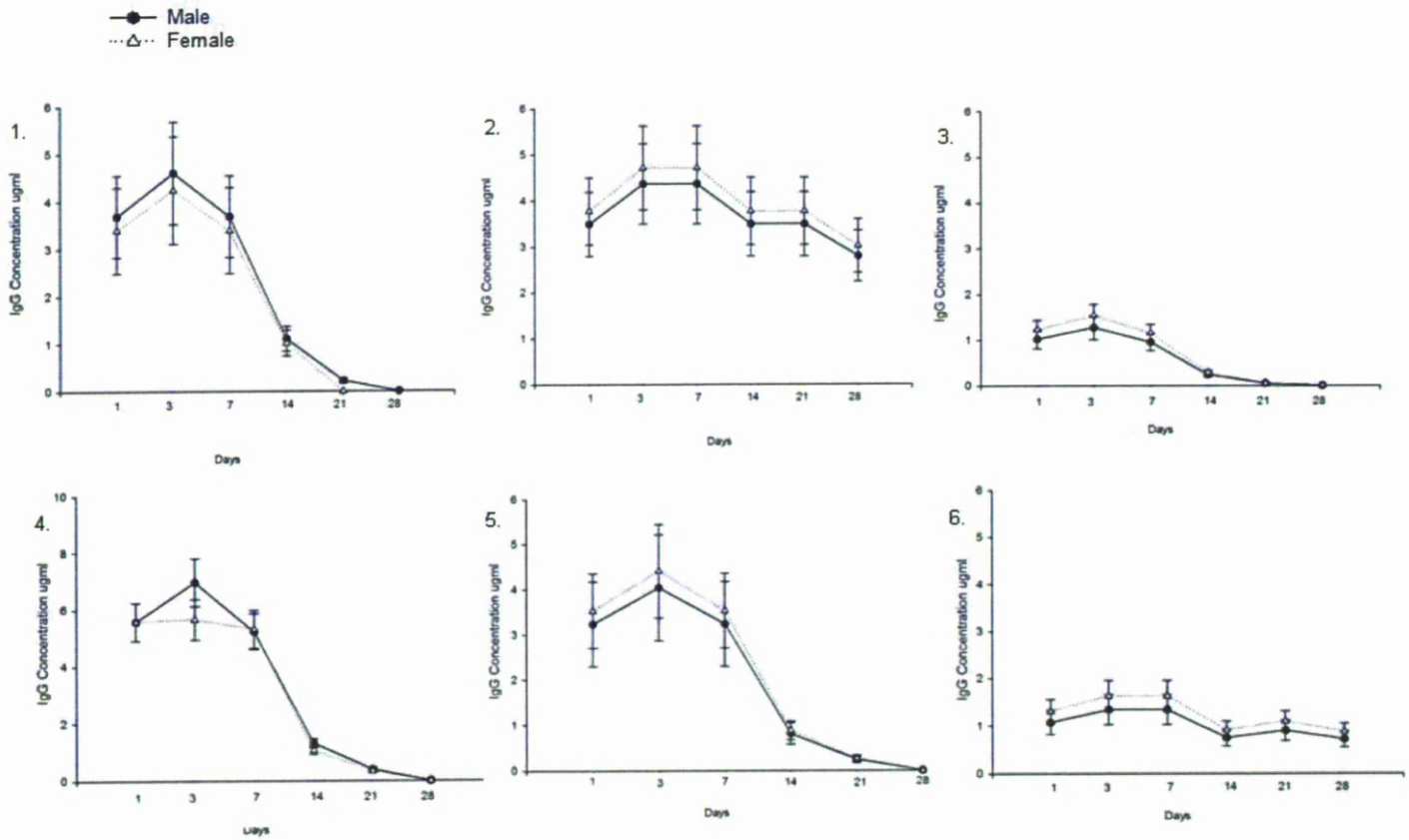

Figure 4.1 The Stability of IgG at room temperature. Samples were collected from male and female volunteers. 1. Drooled oral fluid 2. Orasure 3. Salivette 4. Oracol 5. Seradate 6. Certus. The Bar height represents the mean value ± SE. n = 10

Stability of IgG at 4°C

Measurements for the stability of IgG from collected samples taken using OFCDs and control at 4°C (See figure 4.2).There was no significant differences in levels of IgG concentration between the male and female cohort.

All tested samples were stable post day 7 of the study; this was expected as the samples were maintained at a constant lower temperature.

Post Day 14 the samples treated with protease inhibitor showed signs of degradation this includes the Oracol, Seradate, Salivette and control samples in comparison to the Orasure and Certus.

Post Day 21 sample degradation was more pronounced in the in Oracol, Seradate, Salivette and control samples.

On Day 28 Salivette, Oracol, Seradate and control samples could not be accurately measured as the Levels of IgG were below the detection range of the assay.

There were no significant changes in the levels of IgG in both the Orasure and Certus over the 28 day period.

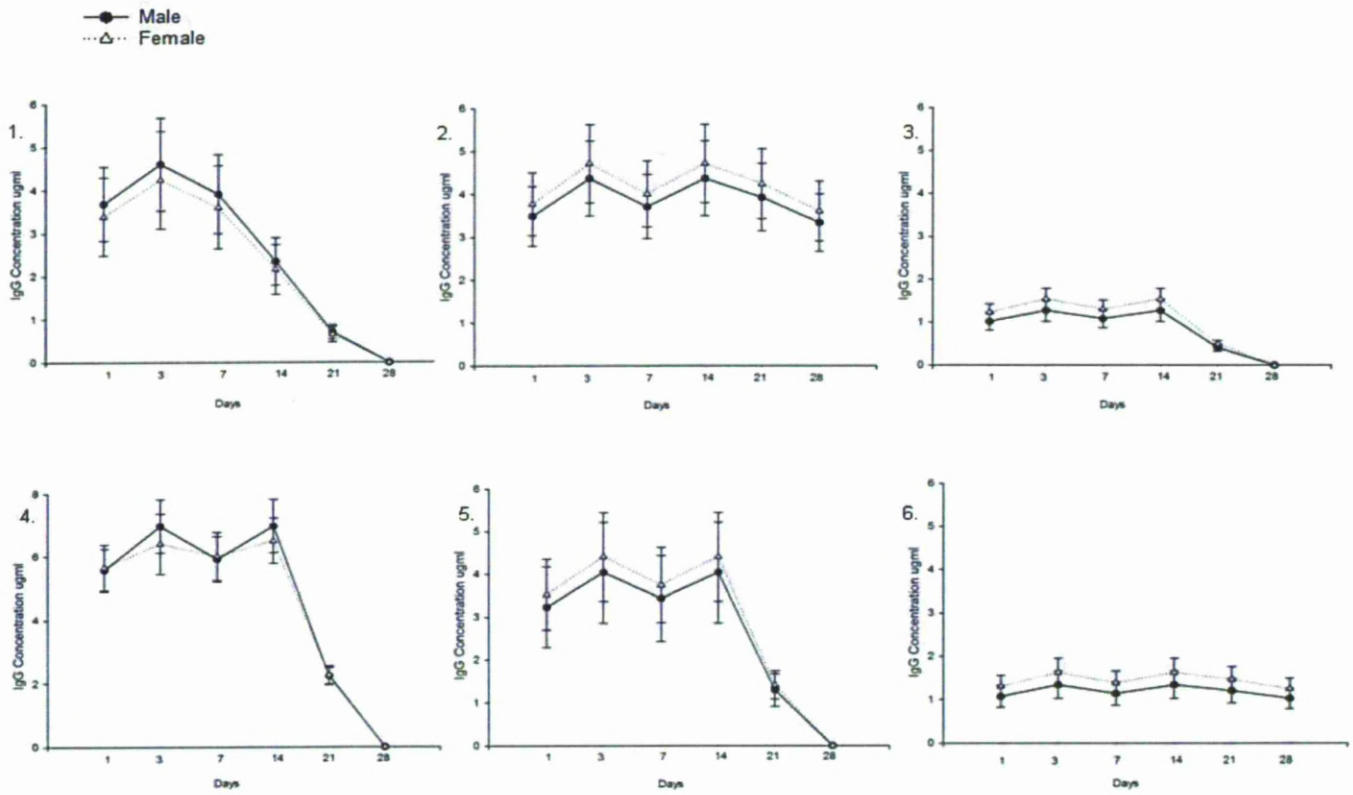

Figure 4.2 The Stability of IgG at 4°C. Samples were collected from male and female volunteers. 1. Drooled oral fluid 2. Orasure 3. Salivette 4. Oracol 5. Seradate 6. Certus. The Bar height represents the mean value ± SE. n = 10

Stability of IgG at -20°C

According to the literature the stability of IgG at -20°C should show a greater resistance to breakdown and exhibit lower degradation. In figure 4.3 the Seradate and control samples showed an initial decline in levels of IgG, but stabilised after day 7 of the study. Both the Certus and Salivette samples maintained levels of stability. There was a steady decline in IgG levels with regards to the Orasure sample in comparison to other temperature, but the discrepancies showed no significant changes overall.

There was a steady decline associated with the Oracol samples, as these samples were not treated any differently to the other test samples, this discrepancy can be associated with possible contamination of the sample.

On day 28 all samples were within the detection range of the IgG assay and showed a greater stability in comparison to 4°C and room temperature.

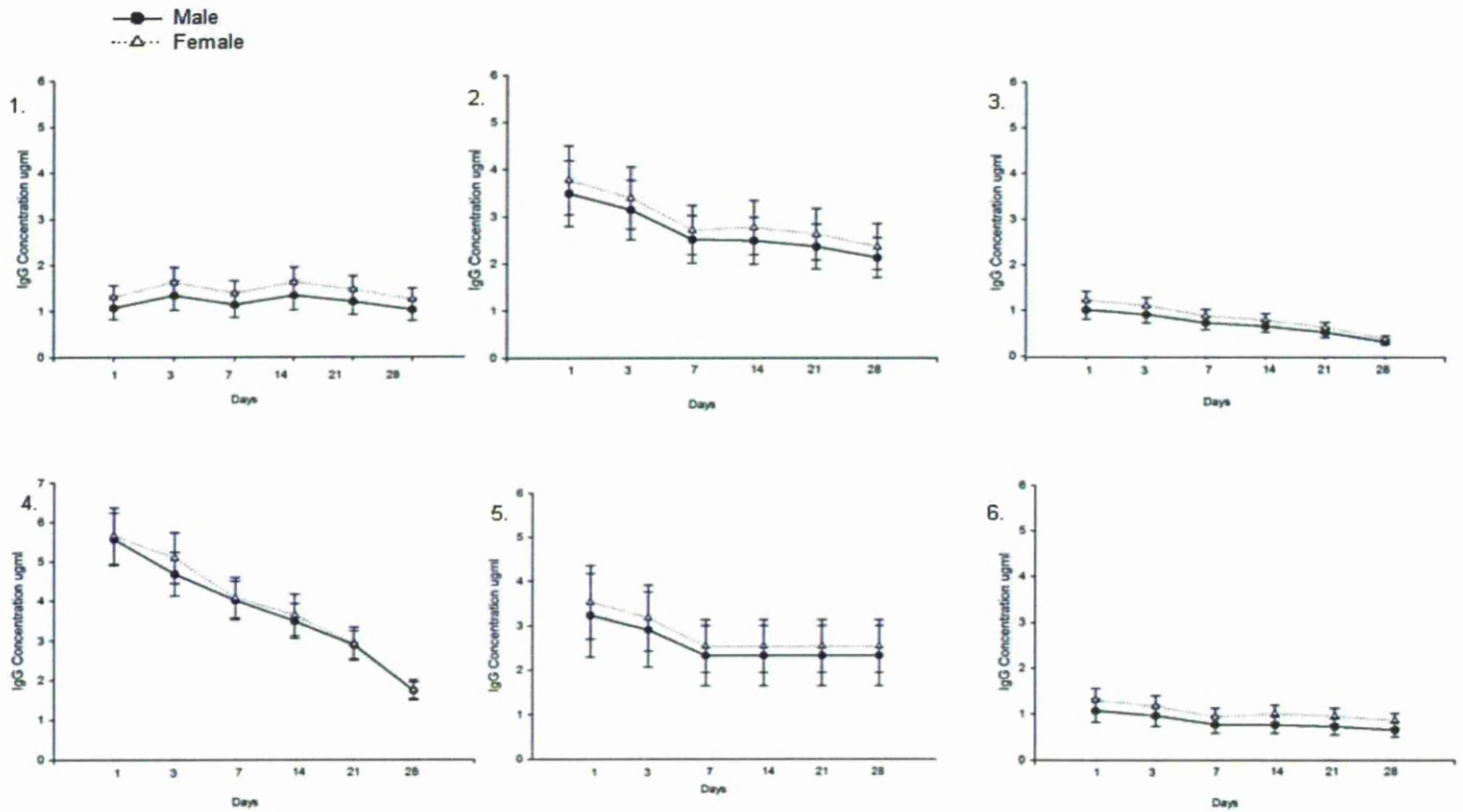

Figure 4.3 The Stability of IgG at -20°C. Samples were collected from male and female volunteers. 1.Drooled oral fluid 2. Orasure 3.Salivette 4. Oracol 5. Seradate 6. Certus. The Bar height represents the mean value ± SE. n = 10

4.5 Monitoring the stability of cotinine at different storage temperatures

Stability of cotinine at room temperature

The stability of cotinine from collected samples, using OFCDs and control at room temperature over a period of 28 days (Figure 4.4).

There were no significant differences in cotinine levels between both the male and female cohort.

There was a slight significant difference in cotinine concentration in both the Oracol and Seradate samples, between time points day 7 and day 28 this could be associated with sample handling and assay inaccuracies. Overall there were no significant changes in the stability of cotinine over the period of the study.

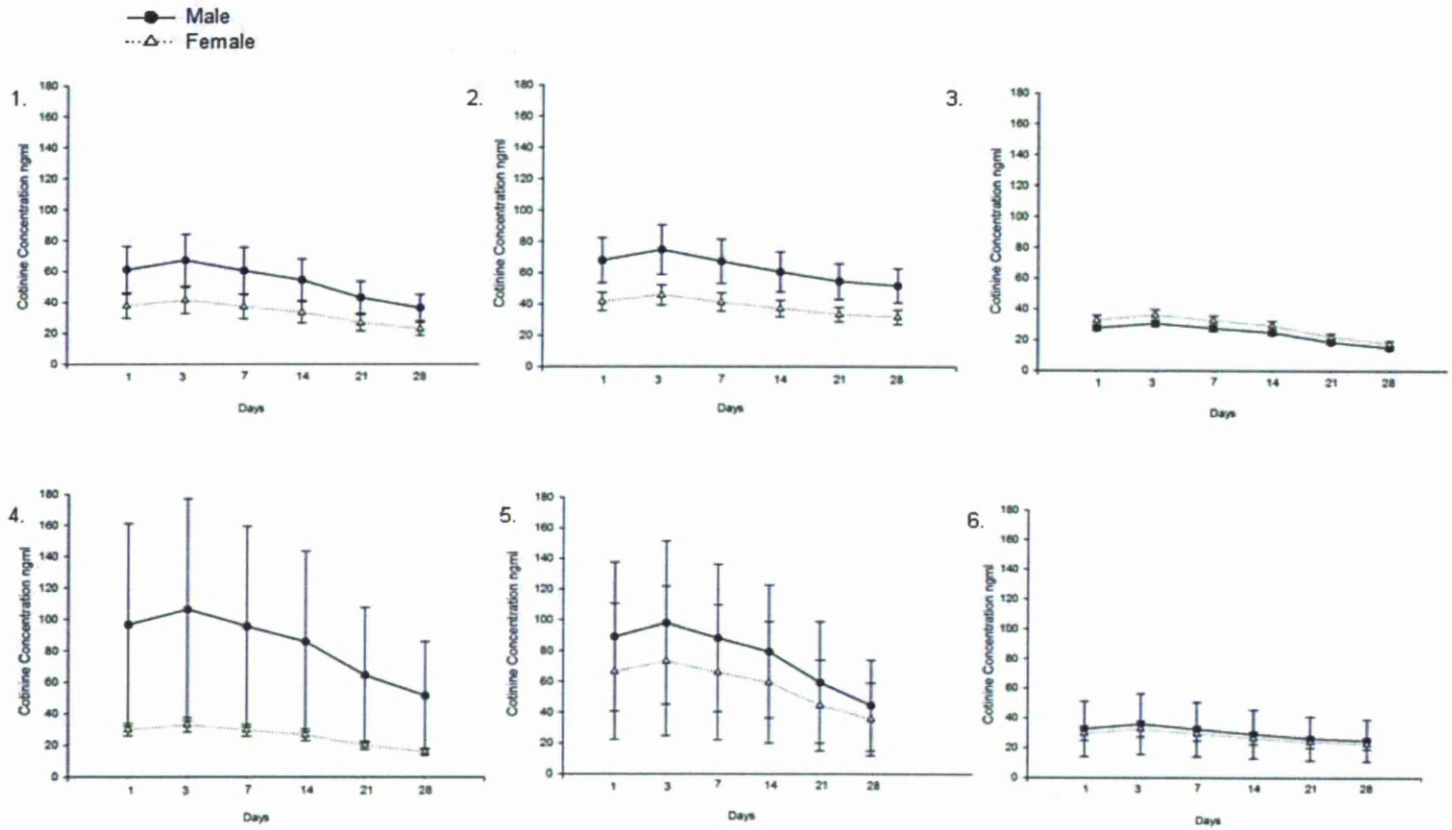

Figure 4.4 The Stability of cotinine at room temperature. Samples were collected from male and female smoking volunteers. 1.Drooled oral fluid 2. Orasure 3.Salivette 4. Oracol 5. Seradate 6. Certus. The Bar height represents the mean value ± SE. n = 10

Stability of cotinine at 4°C

The stability of cotinine from collected samples, using OFCDs and control at room temperature over a period of 28 days (Figure 4.5). There were no significant differences in cotinine levels between both the male and female cohort.

The levels of cotinine concentration were consistent throughout the study period. There were slight significant differences presented by Oracol, Orasure and Seradate. The maintenance of a uniform temperature (4°C) showed greater stability in the levels of cotinine measured throughout the period of study.

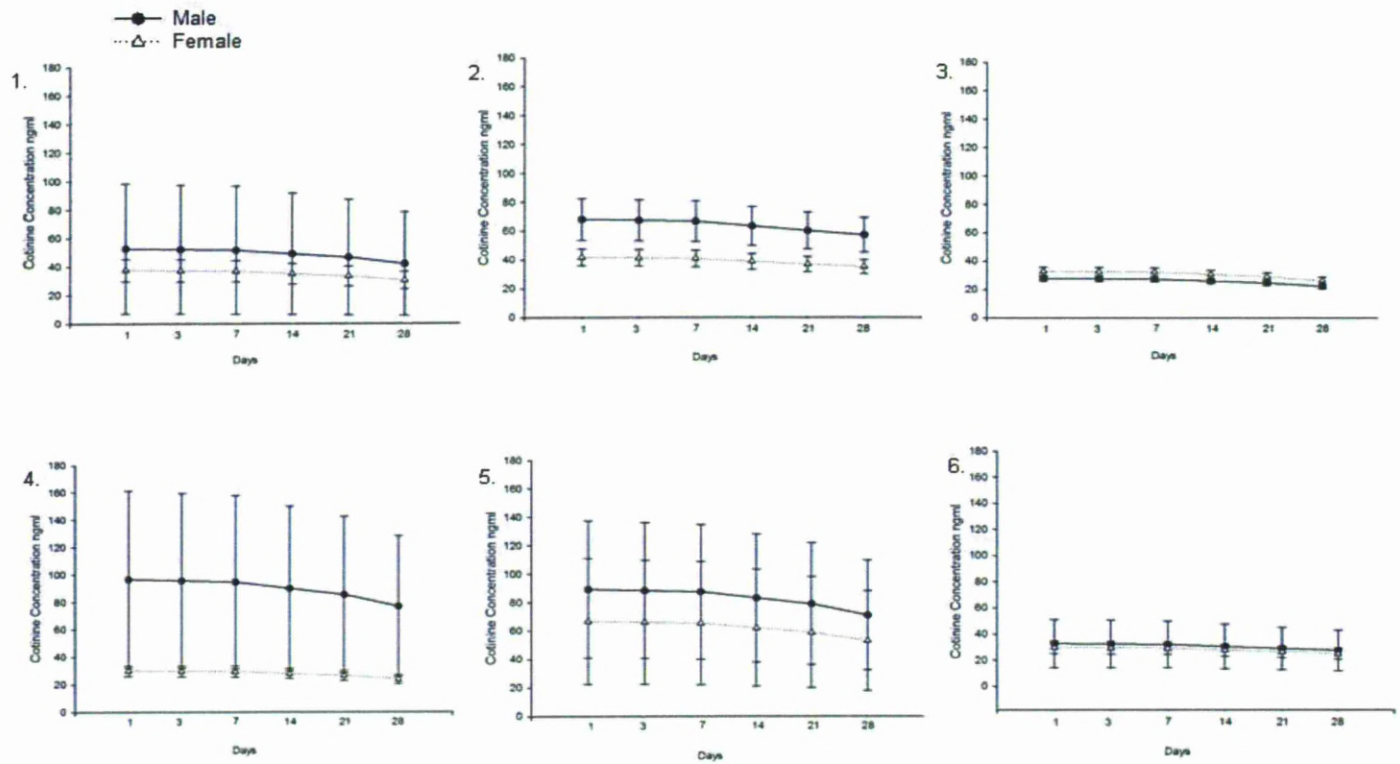

Figure 4.5 The Stability of cotinine at 4°C. Samples were collected from male and female smoking volunteers. 1. Drooled oral fluid 2. Orasure 3.Salivette 4. Oracol 5. Seradate 6. Certus The Bar height represents the mean value ± SE. n = 10

Stability of cotinine at -20°C

The stability of cotinine from collected samples, using OFCDs and control at room temperature over a period of 28 days (Figure 4.6). There were no significant differences in cotinine levels between both the male and female cohort.

The levels of cotinine concentration were consistent throughout the study period. The maintenance of a uniform temperature (-20°C) showed greater stability in the levels of cotinine measured throughout the period of study in comparison to the tested parameters of 4°C and 21°C.

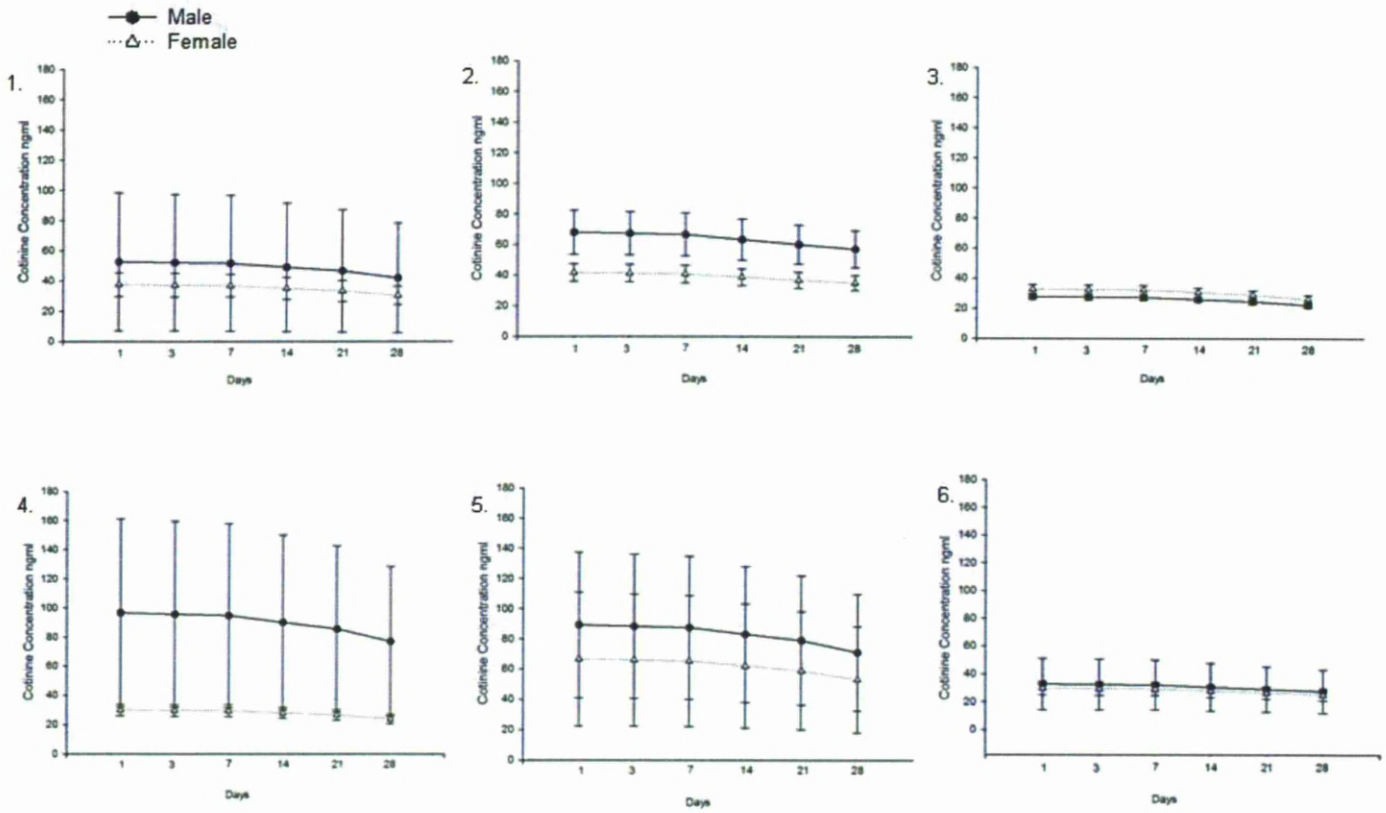

Figure 4.6 The Stability of cotinine at -20°C. Samples were collected from male and female smoking volunteers. 1.Drooled oral fluid 2. Orasure 3. Salivette 4. Oracol 5. Seradate 6. Certus The Bar height represents the mean value ± SE. n = 10

4.6 Discussion

We looked at the stability of oral fluid collected using different OFCDs and drooled oral fluid (Control) over a range of controlled temperatures. To do this the levels of IgG and cotinine were measured. The degradation studies cited in literature only cover shorter time periods between 1-14 days (this is an approximation of the maximum time for a sample to be transported for testing purposes).

The tested samples were subjected to the following temperatures and the stability of IgG and cotinine were monitored

- Room Temperature (21°C)
- Fridge (4°C)
- Freezer (-20°C)

The effects of temperature on the stability of IgG

Room temperature (21°C)

A potential reason for witnessing a degradation of IgG post day 7 in the Oracol, Seradate, Salivette and control can be related to the protease inhibitor used as the effects of protease inhibitors are directed to endogenous proteases present in oral fluid, that are derived from salivary glands and are often directed to serine, cysteine, metallo and aspartic proteases (Kennedy, Davis et al. 1998; Schipper, Loof et al. 2007) and this doesn't account for exogenous proteases released by bacteria within the oral fluid samples. These stipulations coincide with literature in a study conducted by Wong et al that had shown total salivary protein degradation within two weeks with the addition of protease inhibitors. Overall sample integrity can also be affected by sample contamination during and after processing.

Fridge (4 °C)

The samples that were treated with protease inhibitor (Seradate, Oracol, Salivette and control) showed a decrease in stability over a period post 14 days at 4°C. This provides evidence that a lower temperature can improve sample stability until they are affected by bacterial components. Our findings show Post 14 day's oral fluid samples show a decline in IgG concentration with exception of samples collected using the Orasure and Certus, as these OFCDs use anti-bacterial buffers. Oral fluid does contain a mixture of anti-bacterial and anti-proteolytic components that can account for the initial resistance in IgG decline.(Malamud 1997; Esser, Alvarez-Llamas et al. 2008)

Freezer (-20°C)

Chevalier et al stated that oral fluid samples are stable at -20°C in the presence of protease inhibitors (Chevalier, Hirtz et al. 2007). This coincides with our findings as final concentrations of IgG measured in all tested samples showed greater stability at this temperature in comparison to room temperature (21°C) and fridge temperature (4°C).

The effects of temperature on the stability of Cotinine

Cotinine is not metabolised after expectoration, and is not affected by normal oral fluid proteases. A study by Foulds et al on cotinine stability was conducted on samples of untreated saliva collected by cotton roll, their results showed differences in minimal change in cotinine levels over a 12 day period (Foulds, Bryant et al. 1994), all our OFCD samples were monitored for cotinine these showed a steady decline over the period of 28 days, but values were still within limits of the original concentration and would provide correct information for smoking studies. For long term maintenance of oral fluid samples, the ideal temperature of storage would be -20°C and post processing and testing should be conducted within a period of 14 days.

There was a difference in levels of cotinine between our female and male volunteers, as females showed a lower level in comparison to males. However this factor is unrelated to our stability study.

Closing comments

From our data it can be seen that the effects of sample storage are of utmost importance and further testing will be required in the testing of alternate anti-bacterial buffers to find an ideal storage solution for OFCD sample storage. Literature on the stability of oral fluid has suggested potential avenues that can be tested alongside our methods in the study. As protease inhibition within oral fluid is still a problem in oral fluid based studies, potential solutions would be to test the effects of pH on the oral fluid sample by changing acidity and alkalinity (these changes would also need to account for measurements of oral fluid components aside from those tested in this study) or using a higher temperature (boiling

samples). These measures have the potential of denaturing or retarding the proteolytic effects of enzymes present with the test samples. Our study focussed on the stability of IgG and cotinine, further work can be suggestive of measuring other immunoglobulins (salivary IgA and IgM) to compare levels of stability within our chosen temperature range.

The samples stored at room temperature (21°C) and at 4°C all showed signs of an initial increase in IgG concentration that was attributed to evaporation, further work to prevent this would be to wrap sample tubes in parafilm. It can therefore be concluded that oral fluid samples should be kept at temperatures of -20°C or lower to increase sample integrity and standard protocols should be made in accordance to other laboratory methods especially when samples are to be analysed at later date.

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Chapter 5

The effects of smoking on stress markers present in oral fluid

5.1 Introduction

Stress can be termed as a state in which the body's homeostasis is disturbed. The stressor can be of biological, environmental, social, emotional, or of psychological nature. The body's reaction to achieve homeostasis is called stress response. The monitoring of stress markers in oral fluid is documented within the literature, particular attention has focussed on the measuring the levels of salivary alpha amylase and cortisol (Gatti, Antonelli et al. 2009; Nater and Rohleder 2009; Almela, Hidalgo et al. 2011).Stress response is regulated by two primary neuro-endocrine systems, the hypothalamus pituitary- adrenocortical (HPA) system and the sympathetic adreno medullary system. Neuro endocrine markers such as salivary alpha-amylase and cortisol in serum or in oral fluid have an important role in establishing human responses to stressful events. Increases in salivary alpha-amylase upon onset of immediate stress have been tested on stressors such as physical exertion, cold and heat. The use of salivary cortisol measurements within oral fluid testing are well established in comparison to their serum counterpart. Salivary cortisol levels have been used to monitor the activity of the HPA axis. (Gatti, Antonelli et al. 2009; Nater and Rohleder 2009). In Chapter 4 OFCDs were tested to measure cotinine levels, this was used as an indicator of smoking status in individuals, as cotinine levels and inter individual monitoring can provide a more accurate method of establishing number of cigarettes smoked in comparison to self admission(Montalto and Wells 2007; Fu, Fernandez et al. 2009; Almela, Hidalgo et al. 2011). Smoking is a dehabilitating habit,

which affects millions worldwide. Nicotine is the main component of tobacco smoking that leads to addiction. Once nicotine is ingested by smoking or oral administration (tobacco chewing) it is metabolised by the cytochrome p-450 within the liver and is converted into cotinine and nicotine-N-oxide. Cotinine accounts for 80% of the metabolised content of nicotine and has a half-life of up to 24 hours in vivo; this is in contrast to nicotine that exhibits a much shorter half-life of 2 hours.

The monitoring of cotinine is therefore a better indicator for measuring smoking status even when smoking has ceased, as cotinine can be found within oral fluid for periods of up to 10 days and for longer periods within hair and nail samples(Fu, Fernandez et al. 2009; Tzatzarakis, Vardavas et al. 2012).

Smokers report they smoke more under stress and many smokers believe that smoking reduces stress (Hauge, Torgersen et al. 2012), the links between Smoking and the induction of stress are not lucid in the literature. The influence of smoking and its effects on oral fluid markers of stress will be examined. To measure markers of stress alongside smoking the levels of alpha-amylase, cortisol and albumin will also be measured.

5.2 Aims

The aims are to test the Seradate against the OFCDs by measuring stress markers in smoking and non-smoking volunteers. The smoking status of the individuals will be verified by cotinine levels present in the collected samples. The samples will be tested for alpha-amylase, albumin, cortisol and immunoglobulins IgA, IgG and IgM. Based upon smoking status of the individuals, comparisons will be made to the corresponding stress markers. The levels of stress markers in relation to gender will also be tested.

The measurement of the concentrations of albumin and immunoglobulins IgA, IgG and IgM are to provide further information on the effects of smoking on vascular permeability and gum integrity of the smoking and non-smoking cohort. The levels of immunoglobulins in smoking samples will provide an insight into possible compensatory mechanisms that are related to poor oral immunity that results from oral hygiene in smokers.

The samples were collected from a group of male and female volunteers aged between18-30. This group was further sub-divided into smokers and non-smokers.

5.3 Materials & Methods

Measuring levels of cotinine and immunoglobulins in oral fluid

The levels of cotinine and immunoglobulins were measured using the same methodology as stated in chapter 4 using Human IgA/IgM/IgG Elisa quantitation Kit Bethyl Laboratories Inc (Montgomery, USA) and Human Salivary Cotinine detection kit (Salimetrics Europe, Suffolk,UK).

Measuring levels of albumin in oral fluid

The albumin presence in the samples was tested using Human albumin Elisa quantitation kit Bethyl Laboratories Inc (Montgomery, USA) in accordance to manufacturer's instructions. 96 well High Bind flat bottomed plate (Costar Corning Lifesciences, UK) were coated with goat anti-human affinity antibody (Primary Antibody, Bethyl laboratories Inc, USA) .The number of wells used was determined by assay standards, controls and the total number of samples tested (either duplicate or triplicate). 1 µl of capture antibody diluted with 100ul of coating buffer (0.05 M Carbonate-Bicarbonate Sigma Aldrich, UK) administered to each test well. The plate incubated at 21°C for 60 minutes or overnight at 4°C.

The coating buffer was removed by a 3 - step wash (Tecan Columbus plate washer. Austria. Phosphate buffered saline, 0.05% Tween 20).Each step of the wash is involves washing of the wells and aspirating the wells, this process is repeated a total of three times within the 2-step wash process, the plates are then dried by tapping onto blue roll. 200 µl of blocking solution (Phosphate buffered saline, 1% BSA, pH 8.0) was added to each

well. The plate was incubated for 60 minutes at 37°C. After incubation the blocking solution was removed by 3 - step wash (as previously described).

The assay standards used human serum as reference (final serum concentration 1000 ng/ml according to manufacturer's guidelines). 100 µl of sample diluent (Phosphate buffered saline 1% BSA, 0.05% Tween 20, pH 8.0) was added to each well. 100 µl of dilute standard was added to the first well, mixed and 100 µl removed and added to the second well, the process was repeated for the next 7 wells. This produced a standard curve ranging from 1000 ng/ml - 7.8 ng/ml. Oral fluid samples were added these were diluted to optimal concentration to specific wells. The plates were then incubated at 21°C for 60 minutes.

The samples were removed by 5 - step wash (This is a similar process to the 3 - step wash). Secondary HRP detection antibody was added to each test well, this was diluted from stock (1:50,000).100 µl of secondary HRP-detection antibody was added to each well and the plate incubated for 60 minutes. After Incubation the secondary HRP-detection antibodies were removed using a 5-step wash and 100 µl 3, 3', 5, 5'-Tetramethylbenzidine (TMB. Sigma Aldrich) was added to each well. The plate was sealed, covered and placed on a rotating platform for colour development. The development time was between 5-10 minutes, the reaction was stopped by adding 100 µl 4N H_2SO_4 to each test well. The plate was analysed at 450 nm on a spectrophotometer (J Bio LP 400). The sample Immunoglobulin concentrations were calculated from a standard curve using Microsoft Excel (For a detailed list of materials used please refer to appendix B).

Measuring levels of cortisol in oral fluid

To measure sample cortisol levels Human salivary cortisol detection kit (Salimetrics Europe, Suffolk,UK) was used according to manufacturer's instructions. All reagents and plates were brought to room temperature. 1 X Wash Buffer was prepared. 25 µl of standards, controls, and unknowns were placed into appropriate wells. 25 µl of assay diluents was pipetted into an empty well to account for control and non specific binding. The conjugate antibody was diluted 1:1600. 200 µl of conjugate antibody was added to each test well. The plate was mixed at 500rpm for 5 minutes on a plate shaker. The plate was then incubated for 55 minutes at room temperature (21°C). The test samples underwent a four step wash with 1X wash buffer using Tecan Columbus Plate Washer (Austria). The plate was dried and 200 µl of 3, 3', 5, 5'- Tetramethylbenzidine solution was added to each well (Sigma Aldrich, UK). The plate was mixed for 5 minutes at 500 rpm or tapped to mix. The plate was incubated in dark at 21°C for 25 minutes. 50 µl of stop solution was added to each well. The plate was mixed for 3 minutes at 500 rpm on a plate shaker.The plate was analysed at 450 nm on a spectrophotometer (JBio LP 400).The cortisol concentrations in each sample were calculated from a standard curve using Microsoft Excel (for a detailed list of materials used please refer to appendix B).

Measuring levels of alpha-amylase in oral fluid

To measure alpha- amylase in samples the salivary alpha amylase detection kit (Salimetrics Europe, Suffolk, UK) was used according to manufacturer's instructions.
The plate reader was set to incubate at 37°C and to read in center measurements using kinetic mode at one minute and then at three minutes. Samples were diluted 1:10 with assay diluent. The alpha-amylase substrate was heated to 37°C in an incubator. 8 µl of controls or diluted test samples was added to each well. 320 µl of pre-heated alpha-amylase conjugate substrate solution was added each well by multichannel pipette. Measurements were taken at 1 minute and 3 minutes. The 1 Minute reading was subtracted from the 3 Minute reading to give final value for amylase activity (for a detailed list of materials used please refer to appendix B).

5.4 The monitoring of stress markers collected using OFCDs

Comparing cotinine concentration in male and female smokers

The levels of cotinine were measured in the cohort, the group was sub-divided into 2 groups of male and female participants, the values representing the non-smoking individuals were not included as they fell out of the range of the assay and graphical representation would have resulted in a negative plot (see figure 5.1).

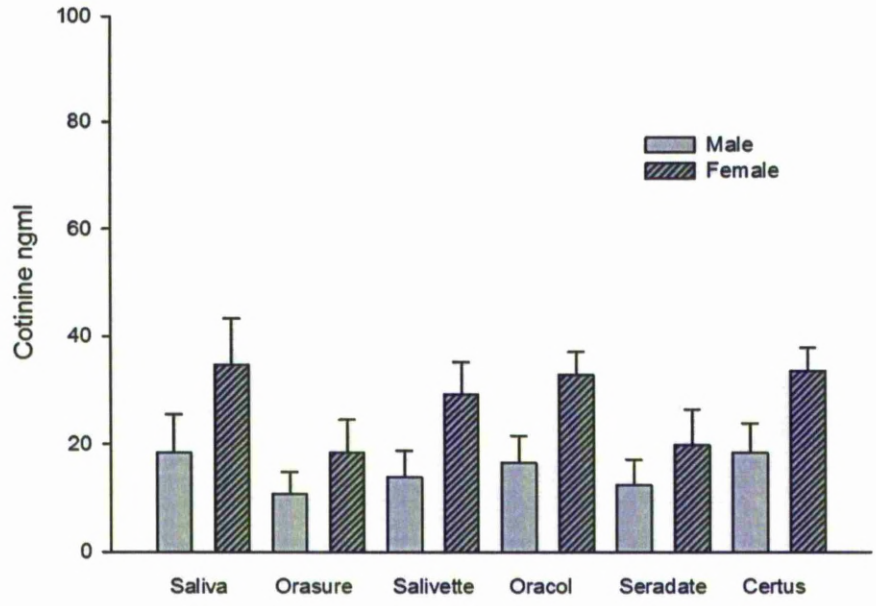

Figure 5.1 The levels of cotinine collected by various collection methods. Bar height the mean value ±SE. n =15.

The data shows that the cotinine values were significantly higher in females than males, possible reasons for this are samples were collected prior to the summer examination period at the university this could account for higher levels of cotinine with response to smoking caused by the onset of stress. The range of cotinine

values is fairly low and although this is indicative of smoking status, individual smoking status was not taken into consideration. The cotinine levels shown are indicative of a light to moderate smoking habit (1-10 cigarettes per day). The consistency of each OFCD in monitoring cotinine is comparable to one another and there are no significant differences in the collection methods the only difference visible are that the female cohort showed higher concentration of cotinine in comparisons to male.

Comparing levels of salivary albumin in males and females

The levels of albumin were measured in the group of smokers and non-smokers (figure 5.2). The levels of cortisol (≈1500 ng/ml) in Orasure, Salivette, Seradate and Certus, these were significantly higher in comparison to the control (≈1200 ng/ml). The Oracol collected the least amount of albumin (≈1000 ng/ml) in comparison to all tested OFCDs and control.

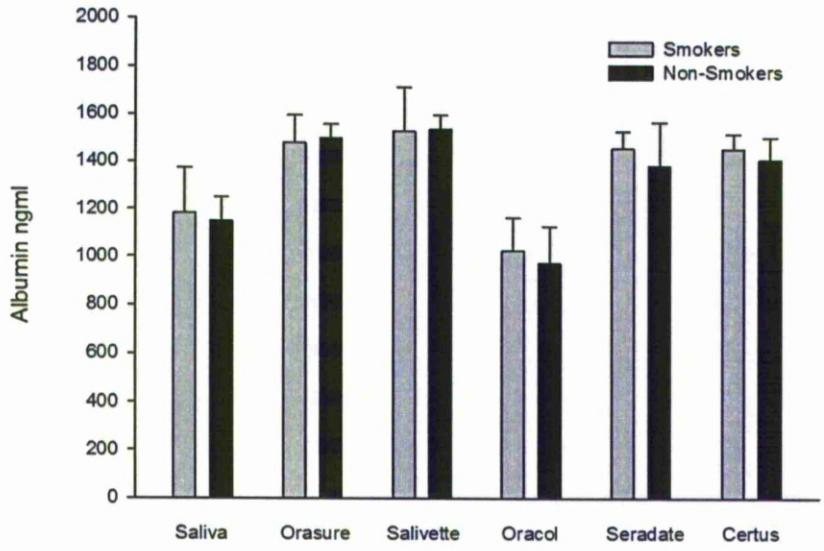

Figure 5.2 Comparing levels of albumin collected between smokers and non-smokers. Bar height the mean value ± SE. n =15.

The levels of albumin were measured in male and females (figure 5.3). The levels of cortisol (≈1500 ng/ml) in Orasure, Salivette, Seradate and Certus, these were significantly higher in comparison to the control (≈1200 ng/ml). The Oracol collected the

least amount of albumin (≈1000 ng/ml) in comparison to all tested OFCDs and control.

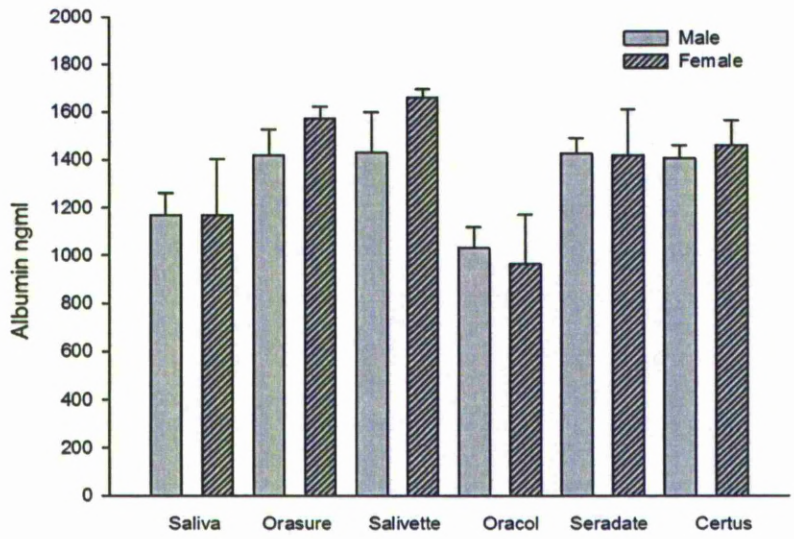

Figure 5.3 Comparing levels of albumin collected between male and female. Bar height the mean value ± SE. n =15.

There were no significant differences between the levels of albumin in neither smoking / non-smoking group nor the male and female group.

The measured levels of albumin showed no significant differences in either of the test groups this suggests that albumin can be used as constant by which to monitor OFCD samples for the involvement of OMT based proteins and its' uses as a complimentary marker for commercial OFCD methods that use IgG as the sole entity for sample validity.

Comparing levels of salivary cortisol in males and females

The levels of Cortisol were measured in the group of smokers and non-smokers (figure 5.4). The levels of cortisol (ranging from 30 ng/ml - 50 ng/ml) in Salivette and Oracol, this was significantly lower in comparison to the control (50 ng/ml - 80 ng/ml) but significantly higher in comparison to Orasure, Seradate and Certus (10 ng/ml -15 ng/ml)

The cortisol levels fluctuated between collection methods but these values were only marginally different when comparing the smokers and non smokers.

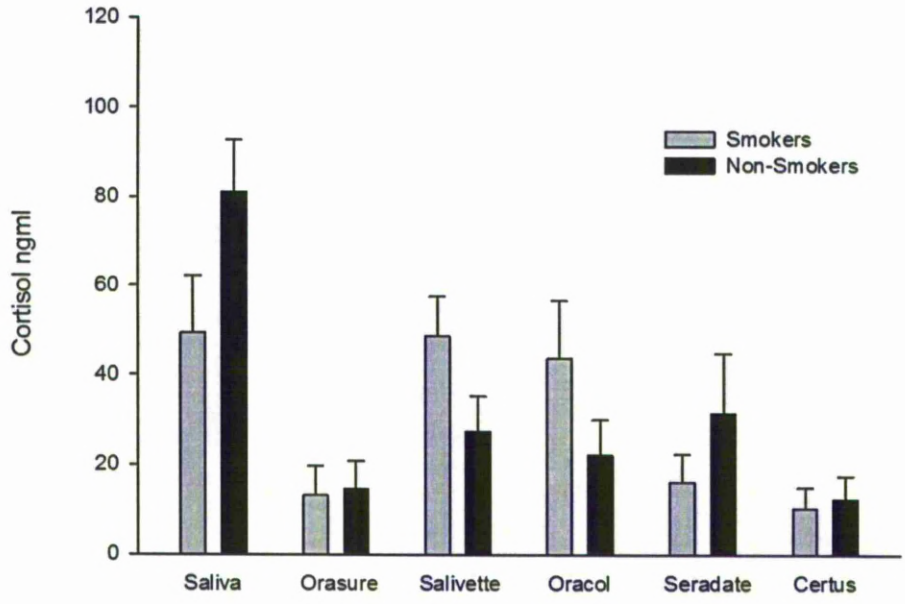

Figure 5.4 Comparing levels of cortisol collected between smokers and non-smokers .Bar height the mean value ±SE. n =15.

The levels of Cortisol were measured in the group of males and females (figure 5.5). The levels of cortisol (ranging from 40 ng/ml-50 ng/ml) in Salivette and Oracol, this was significantly lower in

comparison to the control (60 ng/ml - 80 ng/ml) but significantly higher in comparison to Seradate (20 ng/ml - 40 ng/ml) and Orasure and Certus (≈10 ng/ml). The difference between Males and females showed no significant difference in the levels of cortisol monitored.

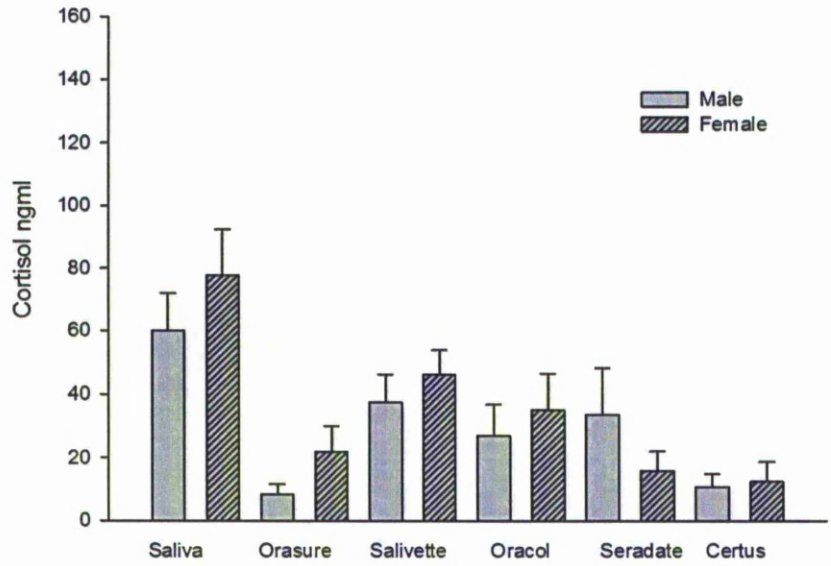

Figure 5.5 Comparing levels of cortisol collected between male and female .Bar height the mean value ±SE. n =15.

The control sample of drooled saliva expressed an excess in cortisol levels measured, this value only varied in slight significance to other samples and was elevated in the non-smoking cohort. The differences in cortisol values by both the Salivette and Oracol could be associated with sample concentration within the material as they differ from the trend seen by all other collection methods.

Comparing levels of salivary alpha-amylase activity in males and females

The activity of alpha-amylase was measured in the group of smokers and non-smokers (figure 5.6). The levels of alpha-amylase activity range from 50 U/ml -90 U/ml in the Salivette which significantly higher in comparison to the control (30 U/ml - 40 U/ml), Orasure, Oracol, Seradate (15 U/ml - 20 U/ml) and Certus (5 U/ml)

There is marginal elevation of alpha amylase in smokers in comparison to the non-smokers group, the only exception to the trend can be seen in drooled saliva sample.

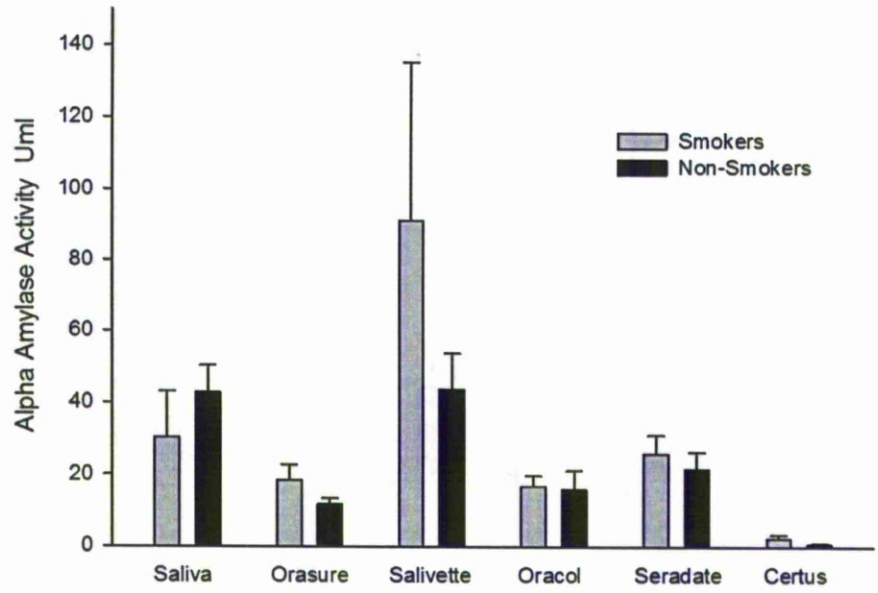

Figure 5.6 Comparing levels of alpha-amylase activity measured between smokers and non-smokers. Bar height the mean value ± SE. n =15

The activity of alpha-amylase was measured in the group of males and females (figure 5.7). The Salivette (50 U/ml) was significantly higher in comparison to the control (30 U/ml - 50 U/ml), Orasure, Oracol, Seradate (15 U/ml – 20 U/ml) and Certus (5 U/ml).

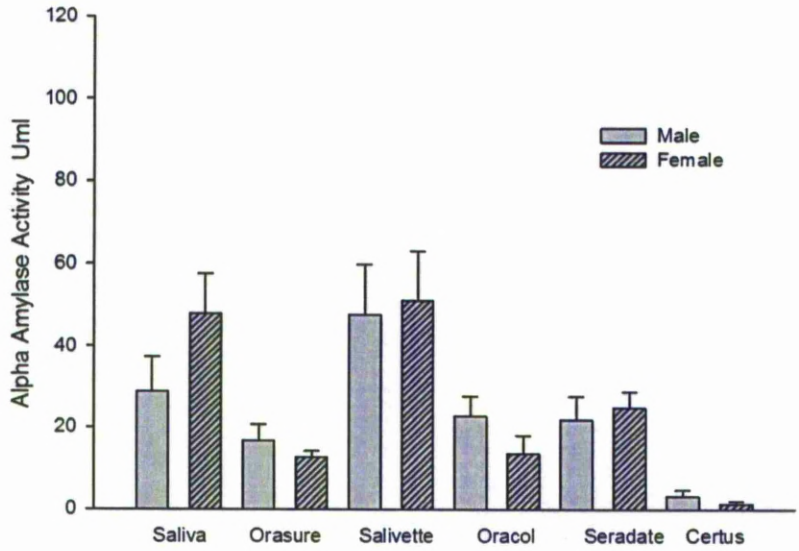

Figure 5.7 Comparing levels of alpha-amylase activity measured between male and female. Bar height the mean value ± SE. n =15

Within the male and female sub-groups there were no significant differences in the levels of alpha-amylase activity amongst the individual .But differences in alpha-amylase activity can be noted in Salivette which showed higher affinity for alpha-amylase release and in contrast the Certus showed the least.

As the values for the remaining OFCDs fall within range of values dictated by drooled saliva (control group), there is a scope for use of OFCDs monitoring alpha-amylase activity.

Comparing levels of salivary immunoglobulins in males and females

Comparing salivary IgA in males and females

The levels of IgA were measured in the group of smokers and non-smokers (figure 5.8).The control (30 µg/ml - 50 µg/ml) was significantly higher in comparison to Orasure, Oracol, Seradate (≈30 µg/ml), Salivette (≈20 µg/ml) and Certus (≈10 µg/ml).

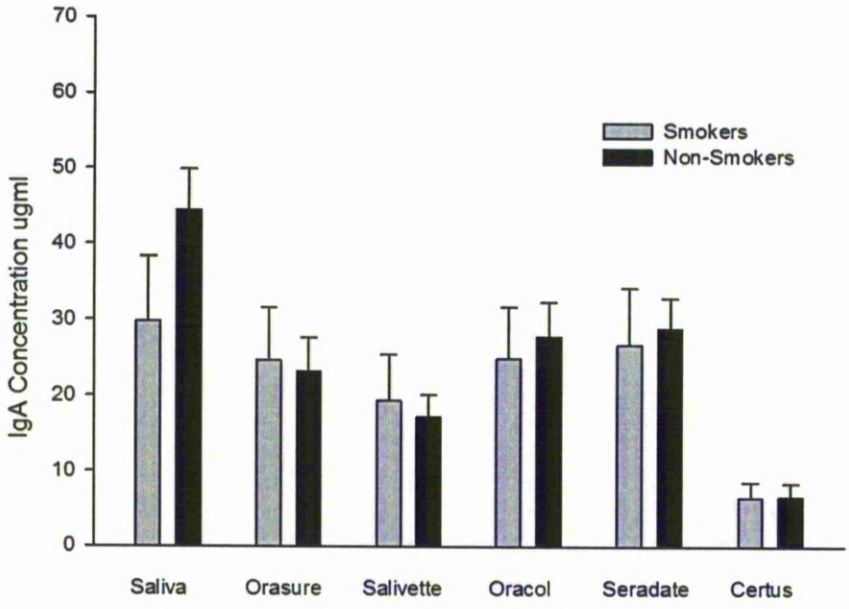

Figure 5.8 Comparing concentration of IgA measured between smokers and non-smokers using various collection methods. Bar height the mean value ± SE. n =15

There was no significant difference between smokers and non-smokers. IgA levels were consistent amongst the OFCD collections; the variation seen in the control group is suggestive of no absorptive material interference in a pure oral fluid sample. The comparatively low levels of IgA presence by the Certus are linked

to previously stated reasons of processing the sample (Certus does not use centrifugation to release collected oral fluid).

The levels of IgA were measured in the group of males and females (figure 5.9). The control (≈35 µg/ml) was significantly higher in comparison to Orasure, Seradate (20 µg/ml - 30 µg/ml), Salivette (10 µg/ml - 20 µg/ml), Oracol (10 µg/ml -15 µg/ml) and Certus (≈10 µg/ml).

The levels of IgA were significantly higher in males in comparison to females by individual OFCDs however no difference was evident within the control group. This could be related to material absorption.

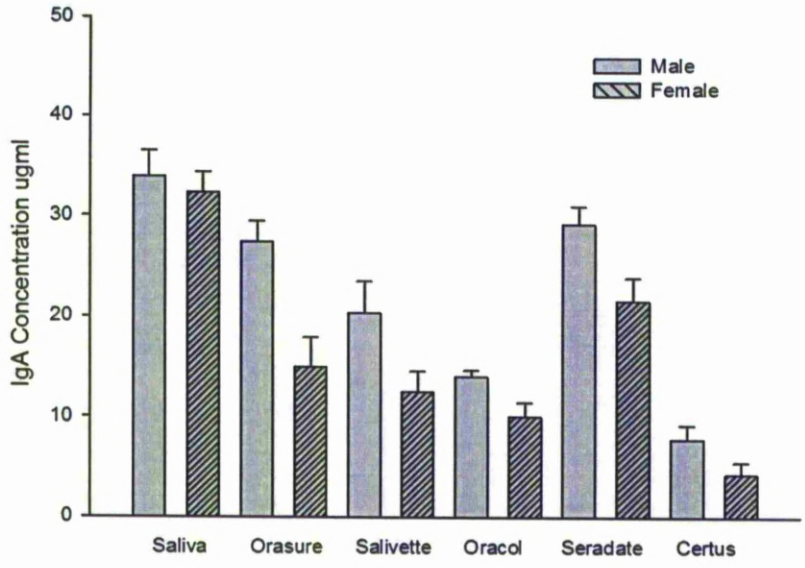

Figure 5.9 Comparing concentration of IgA measured between Males and Females using various collection methods. Bar height the mean value ± SE. n =15

Comparing salivary IgG in males and females

The levels of IgG were measured in the group of smokers and non-smokers (figure 5.10).The Oracol (8 µg/ml) was significantly higher in comparison to control, Orasure, Seradate (≈5 µg/ml), Salivette (≈2 µg/ml) and Certus (≈0.5 µg/ml). There was no significant difference between the smokers and non-smokers among individual OFCDs. The possibility of this occurring is quite unique and will be examined in the discussion.

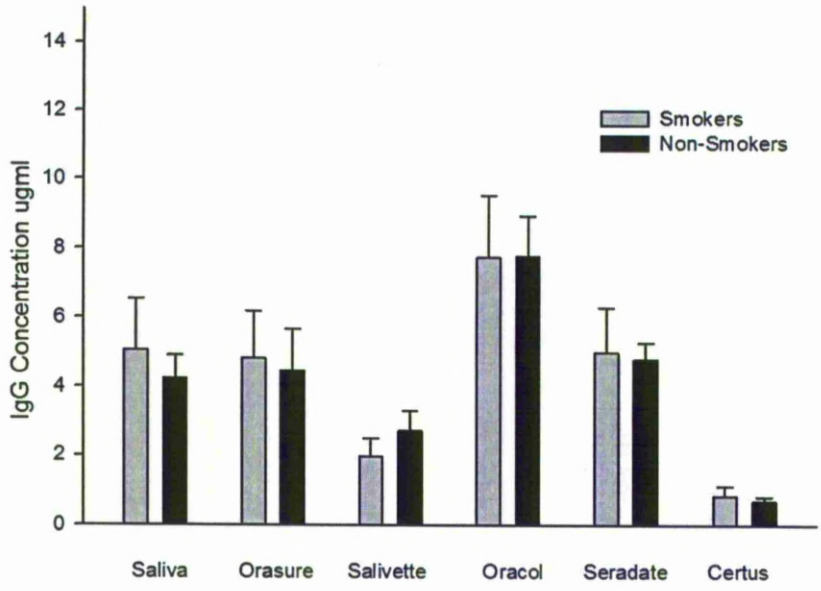

Figure 5.10 Comparing concentration of IgG measured between smokers and non-smokers using various collection methods. Bar height the mean value ± SE. n =15

The levels of IgG were measured in the group of males and females (figure 5.11). The Oracol (4 µg/ml - 7 µg/ml) was significantly higher in comparison to control, Orasure (≈4 µg/ml), Seradate (3 µgml), Salivette (≈2 µg/ml) and Certus (≈0.5 µg/ml).

In both test groups the IgG levels were highest by collection of the Oracol, and lowest levels of IgG were collected by the Certus and Salivette.

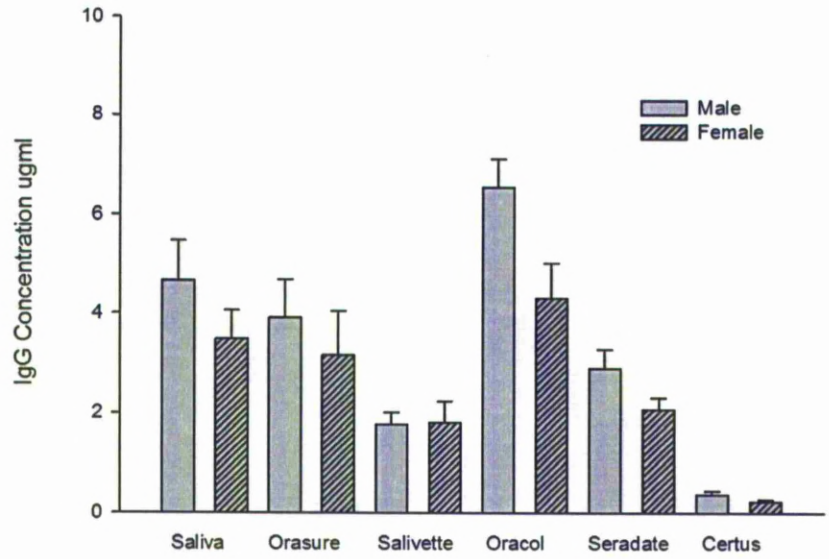

Figure 5.11 Comparing concentration of IgG measured between Male and Female using various collection methods. Bar height the mean value ± SE. n =15

Comparing salivary IgM in males and females

The levels of IgM were measured in the group of smokers and non-smokers (figure 5.12). The Control (2 µg/ml - 6 µg/ml) was comparable Oracol and Seradate. These were significantly higher to Orasure (≈1 µg/ml) and Certus (≈0.5 µg/ml). There was no significant difference between the smokers and non-smokers among individual OFCDs.

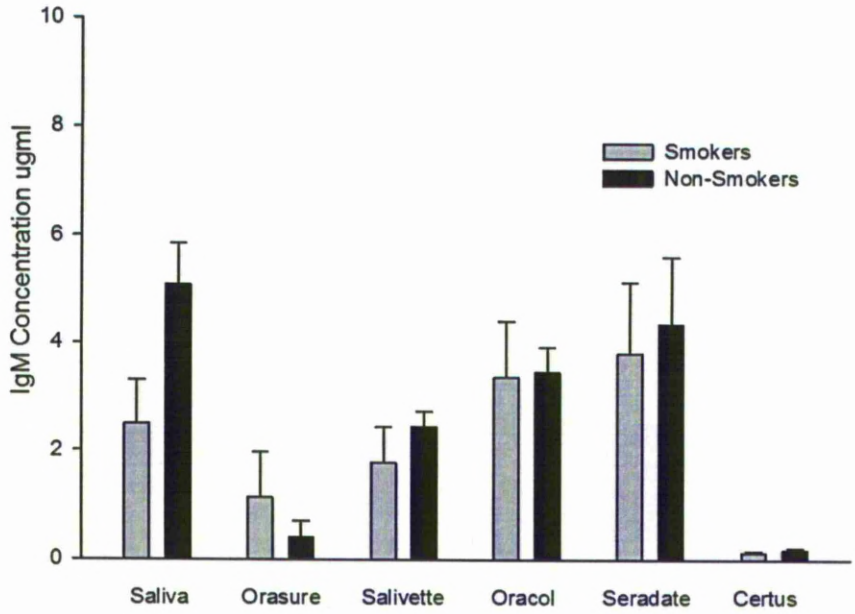

Figure 5.12 Comparing concentration of IgM measured between smokers and non-smokers using various collection methods. Bar height the mean value ± SE. n =15

The levels of IgM were measured in the group of males and females (figure 5.13). The Seradate (2-6 µg/ml) was comparable to the Oracol. These were significantly higher to control (≈3 µg/ml), Orasure, Salivette (1.0 µg/ml -1.5 µg/ml) and Certus (≈0.5 µg/ml). There was no significant difference between the males and females among individual OFCDs.

Lower levels of IgM were presented by the Orasure and Salivette that are potentially related to cotton based interference with the assay.

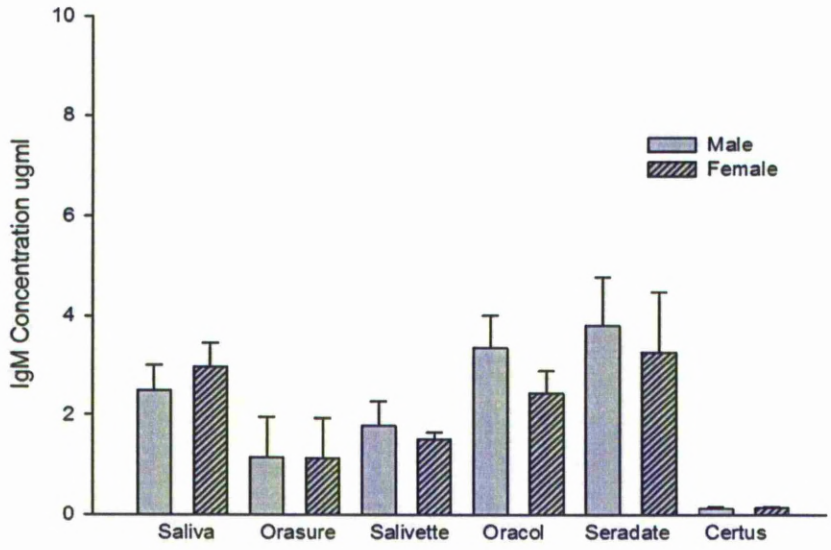

Figure 5.13 Comparing concentration of IgM measured between smokers and non-smokers using various collection methods. Bar height the mean value ± SE. n =15

5.5 Discussion

We aimed to measure variation of oral stress markers on the effects of smoking. Our data was collected amongst a group of males and females and a sub group of smokers and non smokers, both test cohorts showed no significant differences in the monitoring of stress markers and in particular the measurement of stress markers on smoking. The levels of cotinine within the female cohort were higher than those in the male group, alongside the reasons given previously, its noted within the literature that females have a higher metabolism of nicotine and will therefore report higher levels of cotinine in comparison to males (the opposite was observed in our test group in chapter 4)(Benowitz, Lessov-Schlaggar et al. 2006; Berlin, Gasior et al. 2007).

The levels of albumin within the samples served as control in terms of sample validity. The level of albumin in oral fluid is representative of OMT presence. Each of the tested OFCDs including control drooled oral fluid showed respective albumin levels. The levels of albumin between smokers and non-smokers were indifferent, which is supported by the literature, as increases in albumin levels are linked to immunodeficiency.

The measurement of alpha-amylase activity within the groups showed low levels and were within normal ranges of values measured in oral fluid (Nater, Rohleder et al. 2005; Nater and Rohleder 2009). The effects of smoking on levels of alpha-amylase could show levels of increase and decrease, the samples showed lowered salivary alpha amylase have also been associated with smoking caused by inhibition to structure and function of salivary alpha-amylase by acidic aldehydes that are present in tobacco smoke(Granger, Blair et al. 2007). It has also

been reported that smoking has no effect on salivary alpha-amylase release and is attributed to other exogenous factors such as sleep deprivation, body weight and height.

The measurement of cortisol was conducted in the afternoon to allow for controlled values to be taken. The levels of cortisol were predominantly higher in smokers than non-smokers. Literature suggests the possible reason for this is via the activation of HPA via nicotinic receptor activation, thus showing an elevation in cortisol levels (Badrick, Kirschbaum et al. 2007). This however is inconclusive and relation between cortisol levels and smoking are still ambiguous. The possible reasons for these findings are due to limitations within the study. The age group of the tested cohort ranged from student volunteers between the ages of 18-30 and as the smoking activity was not successfully monitored either by the fagerstrom test or self-admission by individuals. An estimation of cigarettes smoked was done measuring cotinine levels against salivary nicotine index. The cohort gave values of low to moderate smoking status. The tested individuals all answered yes to regular dental visits and this provided an insight into oral health of the volunteers. The monitoring of immunoglobulins and possible compensatory mechanisms for oral health could not be suitably established. The limitations to our study was on the induction of a stressor and the effects of smoking, the samples were collected prior to impending summer examinations of the tested individuals, this could have caused the differences measured in values for stress hormones and the link to the effects of smoking could not therefore be well established. As the test was conducted in the course of one day at one set time point, further tests on alternate days prior to the examination may have provided more conclusive data.

By diversifying our age groups to encompass a greater age cohort would have provided a greater level of depth to the study. As the examination of oral health status is monitored in dental publications this could provide screening of test groups to compare smokers with poor oral health to those with good oral health. As most published data focuses on disease groups information is often related to normal and healthy. In the case of our study the groups seem to have merged so no real differences are evident (Bunyaratavej 2006; Vellappally, Fiala et al. 2007).

The collection ability of OFCDs was tested on stress markers present in oral fluid. Studies have shown the use of cotton based OFCDs show alteration to actual levels cortisol and IgA present within normal drooled oral fluid (Strazdins, Meyerkort et al. 2005; Kidd, Midgley et al. 2009; Kozaki, Hashiguchi et al. 2009). This will account for both the Salivette and Orasure OFCDs which showed a lower representation of IgA and cortisol respectively. These findings were inconclusive within our data as levels were comparable with other non-cotton based collection methods.

Closing comments

The effects of absorptive materials has a different effect on final concentrations monitored in assays, these effects will require further standardisation especially in the monitoring of hormones and immunoglobulins alike as they may provide false information to true values present in the human body.

Chapter 6

The screening of oral fluid proteins

6.1 Introduction

The field of proteomics and its advancement in identifying oral fluid proteins have used many analytical methods that include 1D SDS PAGE and 2-D gel electrophoresis protein gels coupled with liquid/gas chromatography mass spectrometry.

The oral fluid matrix is a mixture of protein taken from various salivary glands (the parotid gland, submandibular gland, sublingual gland and minor salivary glands that lie beneath the oral mucosa), OMT and oral micro-biota present within the oral cavity. The quantitation and qualitation of oral fluid proteins will help establish its composition, physiological functions and its potential use as a prognostic and diagnostic fluid. Oral fluid has been quantitated to measure metabolic alterations and pathological conditions that are associated with abnormal concentrations of proteins. (Helmerhorst and Oppenheim 2007; Hu, Loo et al. 2007; Messana, Inzitari et al. 2008).

The composition of oral fluid has been found to differ in its constituents in profiling studies post collection, and shows inter-variability and intra-variability between individuals. This has been associated to the period of sample collection and additional exogenous factors (diurnal changes, eating habits, exercise and oral and systemic health). The protein profiles taken from individual salivary glands also show different levels of proteins, which is evident as either an increase/decrease or lack of protein presentation in comparison to whole oral fluid samples.(Hu, Loo et al. 2007; Messana, Inzitari et al. 2008).

Oral fluid proteins that have been studied include proline rich proteins (PRPs) these account for 60 % of the total oral fluid proteome and are subdivided into acidic, basic and glycosylated groups. Their main functions are the modulation of calcium ions within the oral cavity. They also perform other essential functions within the oral cavity that are yet to be discovered. Histatins are small peptides that possess powerful anti-fungal properties. Statherins are involved in calcium ion homeostasis and teeth mineralisation. Cystatins are inhibitors of cysteine proteases and protect against oral cavity pathogens. Salivary α-amylase accounts for 20% of salivary weight in oral fluid proteins and is the most abundant of the singular proteins in oral fluid. It exhibits a few differences from its pancreatic counterpart, and it is found in oral fluid in various glycol-isoforms .Proteins originating from OMT include serum proteins like salivary IgA, IgG and albumin, proteins of the S100 class and other calcium binding proteins, defensins, thymosin b4 and different peptide hormones.

It is best to view oral fluid proteins within the oral cavity as fish within a tank. The oral cavity collectively recycles proteins from exfoliated cells and oral flora and includes proteins originating from salivary glands and OMT. The distributions of proteins within the oral cavity are dictated by diffusion and concentration gradients. The protein composition in oral fluid is multivariate and the presence of minute quantities of proteins that show non-functional properties may provide answers on oral mucosal degenerative disease and the classification of opportunistic infections within the oral cavity.

To account for changes in protein profiles, research groups have looked at ways to standardize the collected samples; so as to reduce sample variability. To compensate for individual variations

in oral fluid samples, collected samples are often pooled before they are processed.(Hu, Loo et al. 2007). The preparation of Oral fluid samples prior to analysis is dependent on the compounds of interest in a particular study. The profiling of the oral fluid proteins by 2D gel electrophoresis has been subjected to many problems including distortion, smearing and unclear gels. The contributing factors to this are the presence of high molecular weight proteins such as alpha-amylase and albumin. In order to gain clearer protein profiles various methods of sample preparation have been used to limit or remove these components so as to provide a clearer profile and allow the analysis of low abundant proteins, and proteins that are masked by these high molecular weight proteins.(Deutsch, Fleissig et al. 2008; Messana, Inzitari et al. 2008).

Alongside the removal of high molecular weight proteins, the use of gradient 2D gels has also been used to allow for the identification of low molecular weight proteins, 2D gel profiling has range of 120Kda-10 Kda, Proteins that fall above or below this range cannot effectively be monitored. Oral fluid processing is easier to perform than its serum counterpart, and has subsequently led to the discovery of new proteins within oral fluid that are of serum based origin (OMT derivatives); in particular serum based disease markers.(Amado, Vitorino et al. 2005; Helmerhorst and Oppenheim 2007)

6.2 Aims

Our aims are to look at oral fluid proteins collected using the Seradate and OFCDs (used in chapter 4 and 5) in comparison to drooled oral fluid (Control).

1D SDS PAGE protein profiles will be used to screen abundant proteins collected by the Seradate, Orasure, Oracol and Certus against the control. The proteins that show highest intensity (abundance) will be reviewed, to provide qualitative evidence of protein presented by OFCD collection.

2D gel electrophoresis will be used create a protein screening profile of the Seradate OFCD against the control. The samples will be prepared using 3 different methods, to compare any variations in proteins presented between the tested samples. The methods used will be a standard method (developed by Dr. Deborah Ward) and two separate methods cited within literature by Castagnola et al and a new method developed by Deutsch et al. The standard method will function as control in comparison to the other methods.(Deutsch, Fleissig et al. 2008; Messana, Inzitari et al. 2008).

The protein profiles from each sample preparation method will be screened and compared to provide the most suitable method for use in subsequent follow-up experimentation that will be supplemented by the use of mass spectrometry. The proteins will be identified using oral fluid 2D gel profiles taken from literature (Huang 2004).The examination of proteins within the 2D profiles will be directed to the screening of proteins that are of OMT-origin within oral fluid.

6.3 Materials & Methods

Sample preparation for 1D gel electrophoresis

The oral fluid samples tested were collected in the same manner as shown in chapter 4. The protein concentration of each sample was determined using the Biorad protein concentration assay. The samples were precipitated using 10% TCA Acetone overnight. A final concentration of 50 µg/ml for each sample was used. The collected samples were mixed 4:1 with 5 X sample buffer (10% w/v SDS 10nM Dithiothreitol 20% v/v Glycerol 0.2M Tris-HCL 0.05%w/v bromophenol),boiled at 65° C for 10 minutes via heat block.

1D gel electrophoresis

Stacking gel (1.25M Tris-HCl, pH 6.8) and resolving gel (3M Tris-HCl, pH 8.85) preparation can be found in appendix B. Samples were added to wells. 10 µl of ladder marker was placed in the first well followed by 10 µl of each test sample in adjacent wells. Any remaining wells were filled with sample buffer to provide an even load, so as to prevent the gel from smearing.
The gels were run at 100V for 15 minutes 200V until the dye front migrated to the end of the gel. The gel was then removed and stored in container with fixing solution prior to staining. The gels were incubated in fixing solution for 5 minutes at 55°C (shaking). The Fixing solution was removed by aspiration and the gels were incubated in staining solution for 10 minutes at 55°C (shaking). The gels were washed with distilled water and incubated on a shaker in de-staining solution for 10-15 minutes at 55°C. The gels

were then stored in H_2O with acetic acid to prevent drying. The gel image was recorded using a densitometer.

Standard method for sample preparation for 2D gel electrophoresis

The protein concentration of each sample was determined using the Biorad protein concentration assay. The samples were precipitated using 10% TCA Acetone overnight.

Castagnola method for sample preparation for 2D gel electrophoresis (only used on drooled saliva and Seradate)

The protein concentration of each sample was determined using the Biorad protein concentration assay. 10 µl 0.05M 2,2,2-trifluoroacetic acid (TFA) to the sample.The samples were precipitated using 10% TCA Acetone overnight.

Deutsch method sample preparation for 2D gel electrophoresis (only used on drooled saliva and Seradate)

The samples were placed into a 1 ml syringe (Beckmann) with a 0.45mm filter (Millipore). The syringe contained 1g potato starch (Sigma Aldrich). 600µl of distilled water hand pressed into the syringe to saturate the starch. A volume of 1ml saliva (2*500µl aliquots) was hand pressed and filtered. The resultant filtrate concentration was determined using the Biorad protein concentration assay (Biorad Laboratories). The samples were precipitated using 10% TCA Acetone overnight.

2D gel electrophoresis

Preparation and focusing of first dimension gels

3.4 mg of DTT, 2 µl of biolytes added to 1ml IEF rehydration solution was added to a microfuge tube. The tube was placed on a vortex and either 320 µl (17cm strips) or 135 µl (7 cm strips) of the mixture was added to the pre-prepared sample (precipitated protein pellet). The sample/rehydration solution was placed on vortex and left on a rotating platform at 21°C for period of 60-120 minutes (The sample was vortexed at 15 minute intervals). Once dissolved, the sample was centrifuged at 8000g for 5 minutes then applied to Immobilised pH gradient strip (IPG 3-10 NL, Biorad laboratories). Sample solution was then added to the strip holder. (300 µl (17cm strips) or 125 µl (7cm strips) added to the strip holder. The IPG strips were placed gel side down and covered with 1 ml of mineral oil.

Preparation of the 2nd dimension

The glass plates were rinsed using ethanol. The plates were mounted as a sandwich between 2 spacers. The plates were placed within the support construct (placed on a flat surface) and the clamps adjusted to secure construct. Distilled water was used to check for any leaking prior to the addition of the resolving gel.

The resolving gel and stacking gel mixture (detailed materials can be found in Appendix B) was poured between the plates leaving a 2 cm gap at the top. The layer of Saturated Butan-2-ol was then added. Prior to adding the resolving gel is washed using distilled water to remove any saturated butan-2-ol. The stacking gel

mixture is poured on top of the resolving gel. Followed by the addition of saturated butan-2-ol

Preparation of the IPG strips and running 2nd dimension

2*12 ml equilibration buffer added to 240 mg of DTT, 300 mg of IAA, rotated to dissolve. The IPG strips equilibrated for 15 minutes in DTT equilibration buffer, the strip were removed and equilibrated 15 minutes in IAA equilibration buffer.
The saturated Butan-2-ol was removed from the PAGE gel (See Section 2.8.4). The equilibrated IPG strip was placed on top of the stacking gel with good contact on all points. The strip is then covered with melted agarose gel. The Gel units were then assembled and run. For large gels, 15 m/A per gel for 30 minutes until the dye front migrated to the end of the gel. For mini gels 100 V for 15 minutes 200V until the dye front migrated to the end of the gel. Once completed gels removed and stored in a container containing fixing solution prior to staining.

Silver staining of 2D gels

The fix solution was removed using aspiration, and sensitisation solution was added, the gel was left to incubate for 30 minutes on a rotating platform. The Sensitisation solution was removed followed by 3 - step wash using distilled water (allowing an incubation period of 5 minutes between each wash). The distilled water was removed and silver stain solution was added. The container was covered and incubated on rotating platform for 20 minutes. After this period the silver stain solution was removed and the gel washed twice (incubation period of 1 minute).

Development solution was added to the gel, this incubation period can vary between 5-10 minutes until banding can be seen on the gel surface. Stop solution was immediately added to prevent further staining and the gel image was recorded using a densitometer.

6.4 The differences in protein composition of OFCDs using 1D gel Electrophoresis

The protein content of the samples was measured using the Bradford Assay (see materials and methods). The OFCDs used were the Seradate, Orasure, Oracol and Certus. Each of the protein profiles were compared to the control (drooled oral fluid). The initial visualization of the 1D SDS PAGE profiles showed a greater intensity of bands produced by OFCDs in comparison to the control (figure 7.1), the possible reasons for this are associated with retention of water within OFCD swabbing material that caused the samples to show a higher protein concentration in comparison to the control. The Proteins that presented maximum intensity was identified by weight these included albumin, alpha-amylase, Ig-Heavy Chain, Ig-Kappa Light Chain or alpha-amylase fragment, and cystatins. The oral fluid samples were taken from 4 individuals and the collection procedure was conducted in the same manner as described in chapter 3. From the data its can be seen that samples collected all show similar protein profiles of abundant proteins (indicated by arrows on figure 6.1).

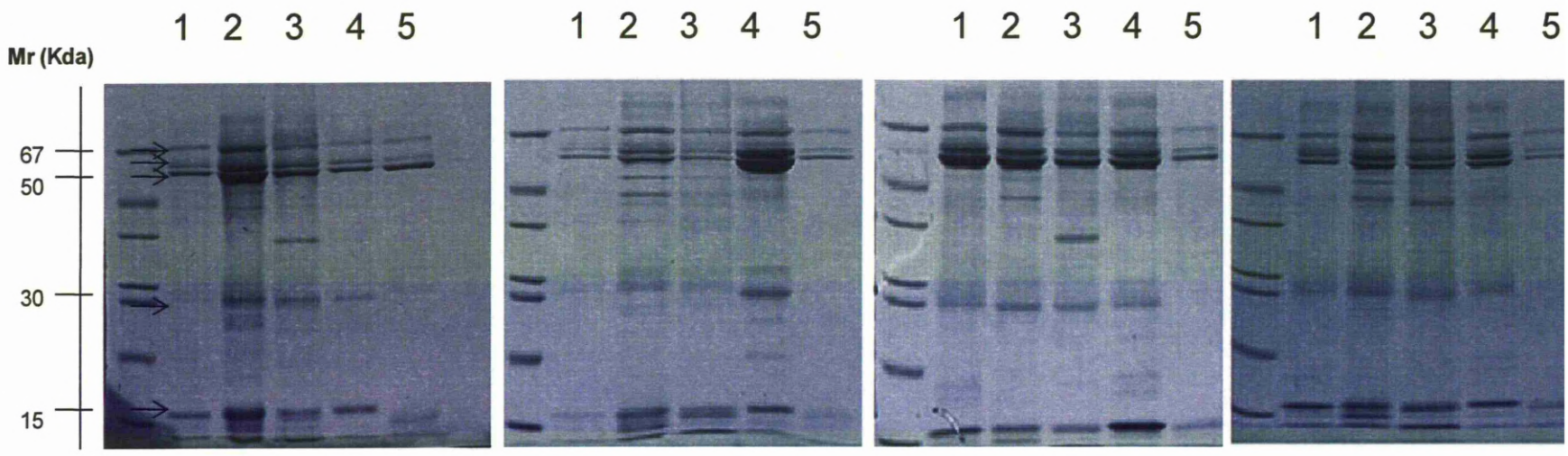

Figure 6.1 1D profile of oral fluid collected by different methods of collection (See materials and methods).

1. Unstimulated Oral Fluid 2. Oracol collection device 3. Orasure oral fluid collection device 4. Seradate Oral fluid collector 5. Concateno Certus collection device. Arrows (top-bottom) albumin,α-amylase,Ig-Heavy Chain, Ig Kappa Light Chain or α-amylase fragment, cystatins.

Comparing intensity levels of albumin

The measurement of albumin was done qualitatively using the mean gray value of each of the bands using Image J software. The values were compared to that of the control and the variation of protein intensity was worked out as a percentage. In figure 6.2 the Orasure expressed a 25% increase in albumin collection Seradate (10%) and Oracol (10%) in comparison to the control. The Level of albumin collected by the Orasure was significantly higher in comparison to the other collection devices .The Certus expressed albumin levels lower than that collected from the control, and is associated with processing of the sample after collection. The Seradate expressed no significant differences against the Oracol, both the Seradate and Oracol showed significantly lower values of Albumin in comparison to the Orasure.

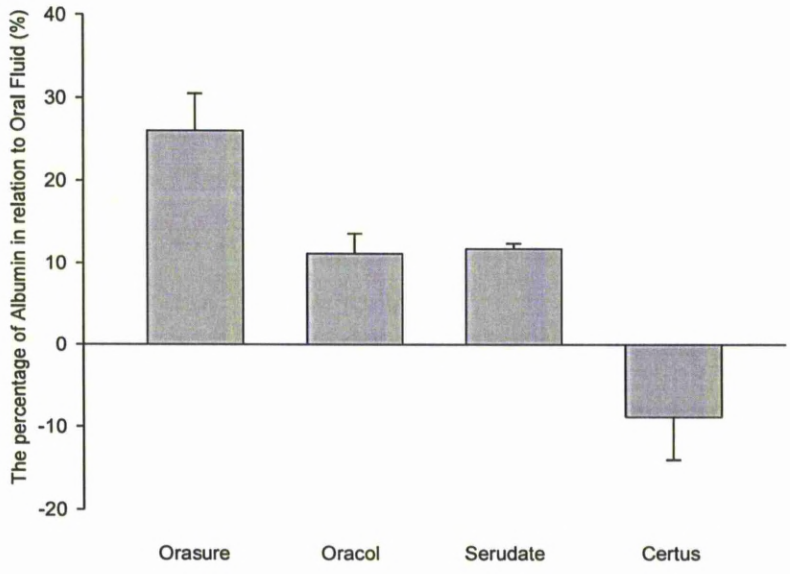

Figure 6.2 The level of albumin detected in relation to oral fluid. The mean grey value for each band was compared with that of expectorated oral fluid. The percentage value is a quantify intensity expressed in each band and corresponds to level of protein visualized. Error bars show ± SEM. n =4.

Comparing intensity levels of alpha-amylase

The intensity levels of alpha-amylase were compared from the 1D profile (Figure 6.3) the findings show that the Orasure and Seradate OFCDs show a 20% increase in alpha-amylase collection, Oracol (10%) compared to OF. There was no statistically significant difference in the intensity of the protein between the Orasure, Oracol and Seradate. The Certus showed lower levels of alpha-amylase levels in comparison to the control.

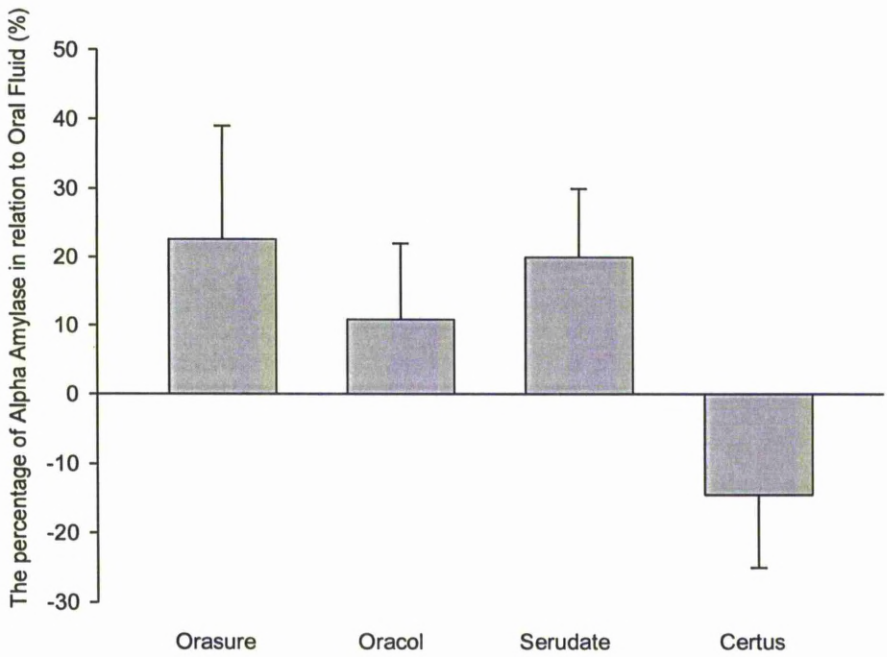

Figure 6.3 The level of alpha-amylase detected in relation to oral fluid. The mean grey value for each band was compared with that of expectorated oral fluid. The percentage value is to quantify intensity expressed in each band and corresponds to level of protein visualized..Error bars show ±SEM. n =4

Comparing intensity levels of Ig-Heavy chain

The Intensity levels of Ig-Heavy chain were compared with the 1D profile (see figure 6.4) the percentage difference in comparison to the control showed an increase in Ig-Heavy Chain collection by Orasure (20%) Seradate (15%), Oracol (10%).There was statistically no significant difference in the Intensity levels of the protein between the Orasure, Oracol and Seradate. The Certus showed statistically significant lower Intensity levels of Ig-Heavy Chain in comparison to control.

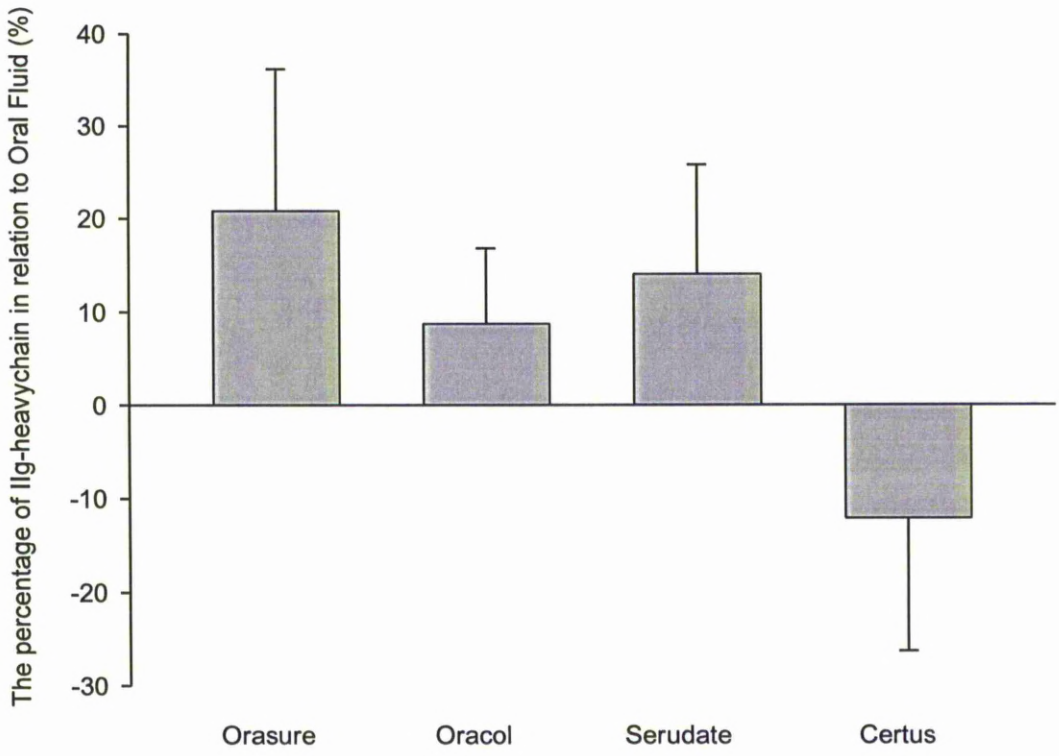

Figure 6.4 The level of Ig-Heavy chain detected in relation to oral fluid. The mean grey value for each band was compared with that of expectorated oral fluid. The percentage value is to quantify intensity expressed in each band and corresponds to level of protein visualized. Error bars show ± SEM. n =4.

The Intensity levels of Ig- Kappa Chain/Fragment of alpha-amylase detected in the 1D gel profile in comparison to the control (see figure 6.5) The percentage in difference of intensity measured against the control Orasure (20%) Seradate (10%), Oracol (8%). There was statistically no significant difference between the Orasure, Oracol and Seradate. The Certus showed statistically significant lower Intensity levels of Ig-Heavy Chain in comparison to control.

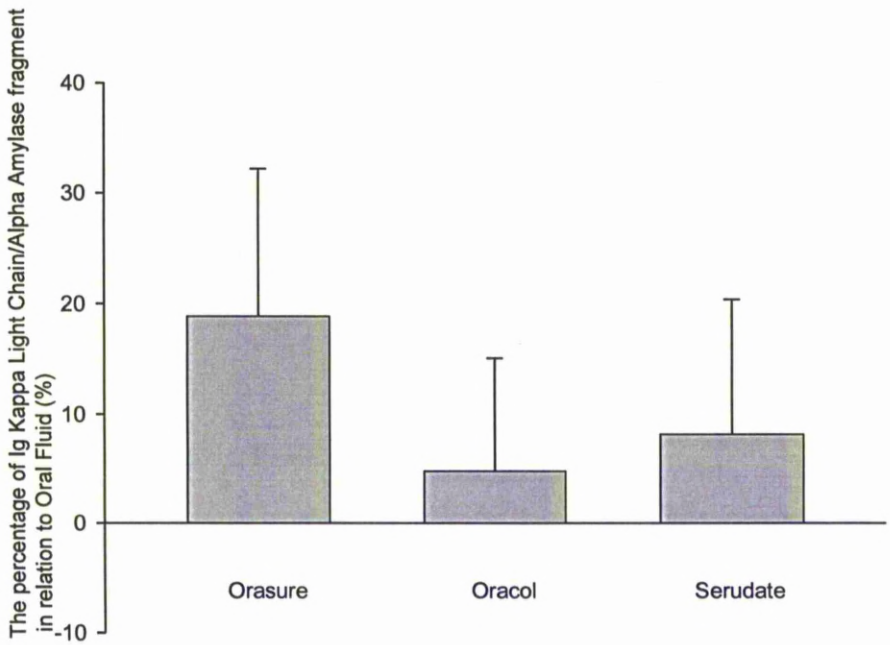

Figure 6.5 The level of Ig- Kappa Light chain/ alpha-amylase fragment detected in relation to oral fluid. The mean grey value for each band was compared with that of expectorated oral fluid. The percentage value is to quantify intensity expressed in each band and corresponds to level of protein visualized. Error bars show ± SEM. n =4.

Comparing levels of cystatins

The Intensity levels of cystatins detected in the 1D gel profiles (see figure 6.6) show that the Orasure, Seradate and Oracol expressed a 10% increase in cystatins collection in comparison to the control. The Orasure, Seradate and Oracol showed no statistically significant difference in the expression of the protein .The Certus showed statistically significant lower Intensity levels of Ig-Heavy Chain in comparison to control.

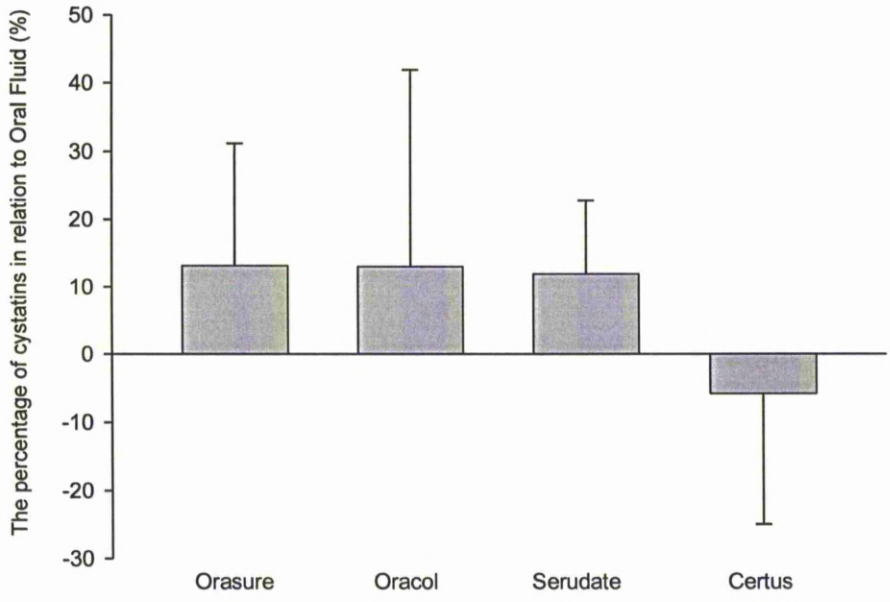

Figure 6.6 The level of cystatins detected in relation to oral fluid. The mean grey value for each band was compared with that of expectorated oral fluid. The percentage value is to quantify intensity expressed in each band and corresponds to level of protein visualized. Error bars show ± SEM n =4.

6.5 2D gel using different methods of sample preparation to show differences in protein profiles of Seradate against control

The Oral fluid samples collected using the Seradate was compared against the control to examine differences in protein profiles using 2D gel electrophoresis. The samples were collected from 5 individuals and pooled to reduce variability.

Three different sample preparation methods were used

1. The standard method was used as control (Developed by Dr. Deborah Ward. University of Liverpool)

2. The Castagnola Method. The addition of 2,2,2-trifluoroacetic acid (TFA) to the sample, this is expected to inhibit a number of degradation enzymes in the sample. The method is used to remove high molecular weight proteins such as albumin and α-amylase, to help produce a clearer 2D profile of oral fluid proteins(Castagnola, Inzitari et al. 2004; Messana, Inzitari et al. 2008).

3. The Deutsch Method removes alpha amylase by affinity adsorption to potato starchto help produce a clearer 2D Profile of oral fluid proteins (Deutsch, Fleissig et al. 2008).

The different sample preparation methods were tested for any reductions in protein intensity between the Seradate and the control, and to examine any interference within the gels, that may cause distortions or smearing in the 2D profiling of oral fluid proteins.

The main proteins of interest were identified using current literature as a reference(Huang 2004) (see table 6.1) and marked

on figures 6.8, 6.10 and 6.12. These were used to measure differences in protein profiles between Seradate and control. Qualitative measurements of Intensity were taken for proteins of interest using the mean grey area using Image J software. The difference in protein intensity was recorded as a percentage to show either an increase or decrease in protein intensity collected by comparing the Seradate against the control.

Area	Protein
1	Albumin*
2	Alpha-Amylase
3	Prolactin Inducible Protein
4	Ig-Kappa Chain*
5	Cystatins
6	G3P dehydrogenase
7	Haemaglobin Beta Chain*

Table 6.1 Predominant proteins expressed in oral fluid and seen in figures 6.8, 6.10 and 6.12. *OMT derivative markers

2D Gel comparisons of Seradate against control using the standard method.

The 2D gel profiles were run using the standard sample preparation method. The differences in protein intensity were measured for the 7 proteins of interest. The data collected (see figure 6.7 and 6.8) show elevations in levels of albumin, prolactin Inducible protein and hemoglobin beta chain. The involvement of these proteins shows that the Seradate has an affinity for OMT derived proteins. The reductions in levels of α-amylase, Ig-heavy chain, and cystatins show possible retention of components within the absorbent material of the Seradate.

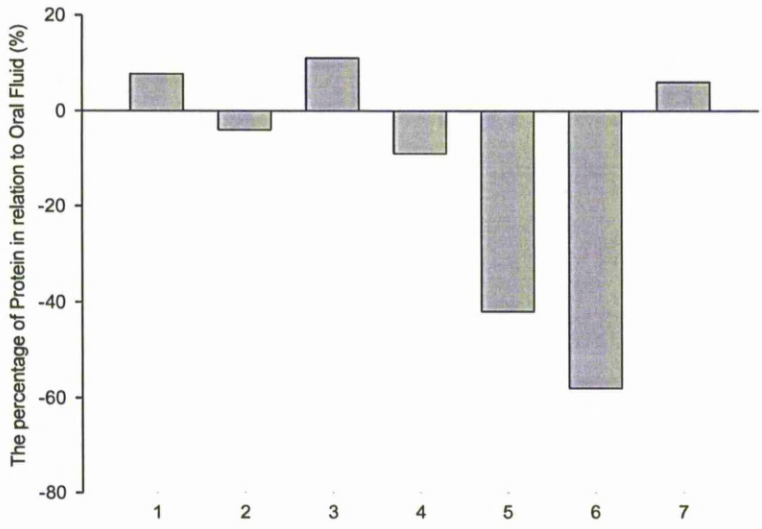

Figure 6.7 The percentage of Seradate proteins in relation to control (the standard sample preparation method). 1. Albumin 2.Alpha-amylase 3.Prolactin Inducible Protein 4.Ig-Kappa Chain 5.Cystatins 6. G-3-P dehydrogenase 7. Haemoglobin Beta Chain. The samples were pooled from 5 individuals. The SEM is too small to show graphically ≤0.5%.

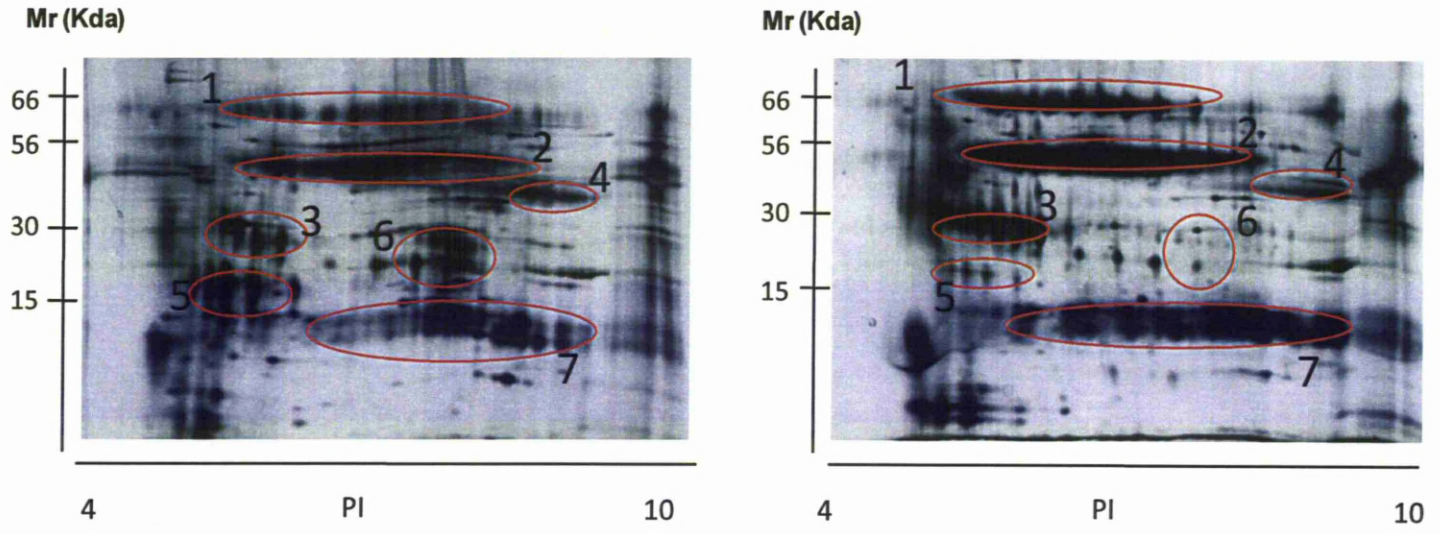

Figure 6.8 2D SDS PAGE Gels (standard method of sample preparation) **LHS** Control **RHS** Seradate Oral Fluid.

Highlighted regions 1. Albumin 2.α -amylase 3.Prolactin Inducible Protein 4.Ig-Kappa Chain 5.Cystatins 6. G-3-P dehydrogenase 7. Haemoglobin Beta Chain.

2D Gel comparisons of Seradate against control using the Castagnola method.

The 2D gel profiles were run using the preparation method suggested by Castagnola et al (Castagnola, Inzitari et al. 2004; Messana, Inzitari et al. 2008). The variation of protein intensity was measured for the 7 proteins of interest. The data collected (see figure 6.9 and 6.10) shows elevation in levels of prolactin inducible protein and cystatins. The involvement of these proteins shows that Seradate shows elevated levels of OMT derived constituents. The low levels shown by other proteins may have been caused by the method and the possible retention of sample with the absorbent material of the Seradate.

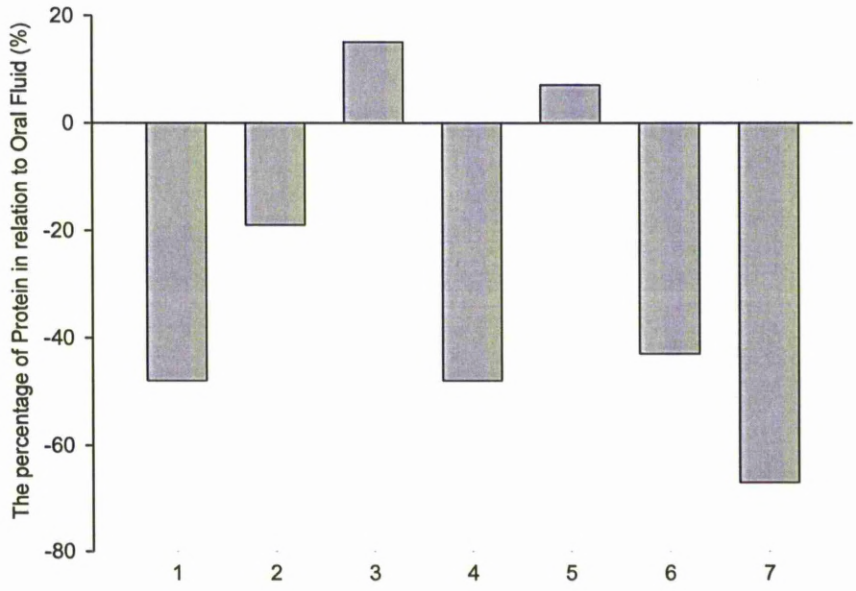

Figure 6.9 The percentage of Seradate proteins in relation to control using Castagnola sample preparation method. 1. Albumin 2.Alpha-amylase 3.Prolactin Inducible Protein 4.Ig-Kappa Chain 5.Cystatins 6. G-3-P dehydrogenase 7. Haemoglobin Beta Chain.The samples were pooled from 5 individuals. The SEM is too small to show graphically ≤0.5%.

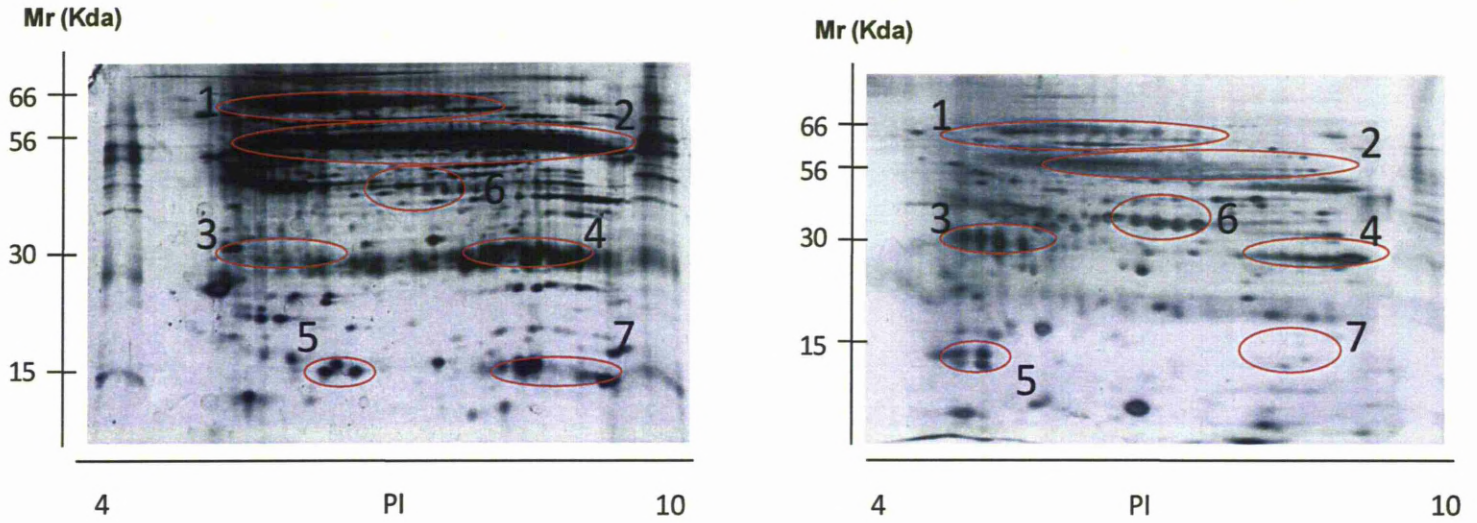

Figure 6.10 2D SDS PAGE Gels (Castagnola sample preparation) **LHS** Control **RHS** Seradate Oral Fluid. Highlighted regions

1. Albumin 2.Alpha-amylase 3.Prolactin InducibleProtein 4.Ig-Kappa Chain 5.Cystatins 6. G-3-P dehydrogenase

7. Haemoglobin Beta Chain.

2D Gel comparisons of Seradate against control using the Deutsch method.

The 2D gel profiles were run using the preparation method suggested by Deutsch et al (Deutsch, Fleissig et al. 2008).The variation of protein intensity was measured for the 7 proteins of interest. The data collected (see figure 6.11 and 6.12) shows increased intensity of all proteins of interest, when comparing the Seradate against control. The method of sample preparation shows a reduction in proteins present within the control sample, there is a comparable reduction in protein intensity in the Seradate gel. Possible reasons for this are a more concentrated level of high molecular weight proteins present within the Seradate sample that could not be effectively cleared by the sample preparation method.

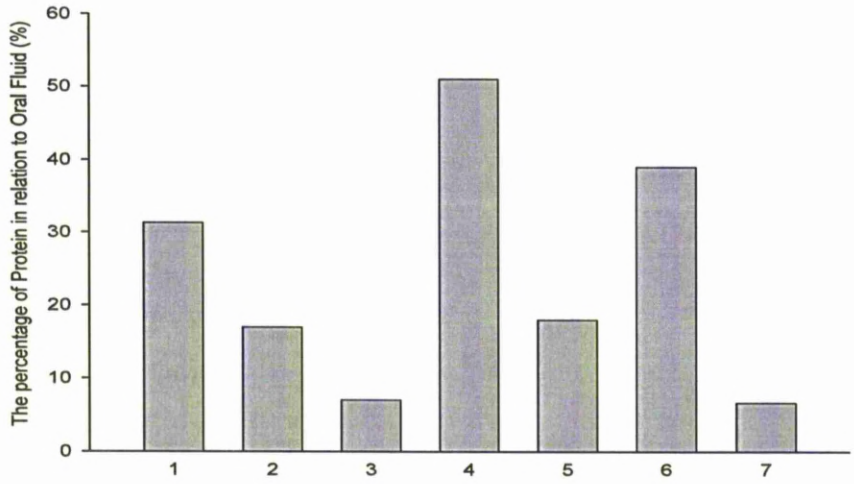

Figure 6.11 The percentage of Seradate proteins in relation to control using Deutsch sample preparation method. 1. Albumin 2.Alpha-amylase 3.Prolactin Inducible Protein 4.Ig-Kappa Chain 5.Cystatins 6. G-3-P dehydrogenase 7. Haemoglobin Beta Chain. The samples were pooled from 5 individuals. The SEM is too small to show graphically ≤0.5%.

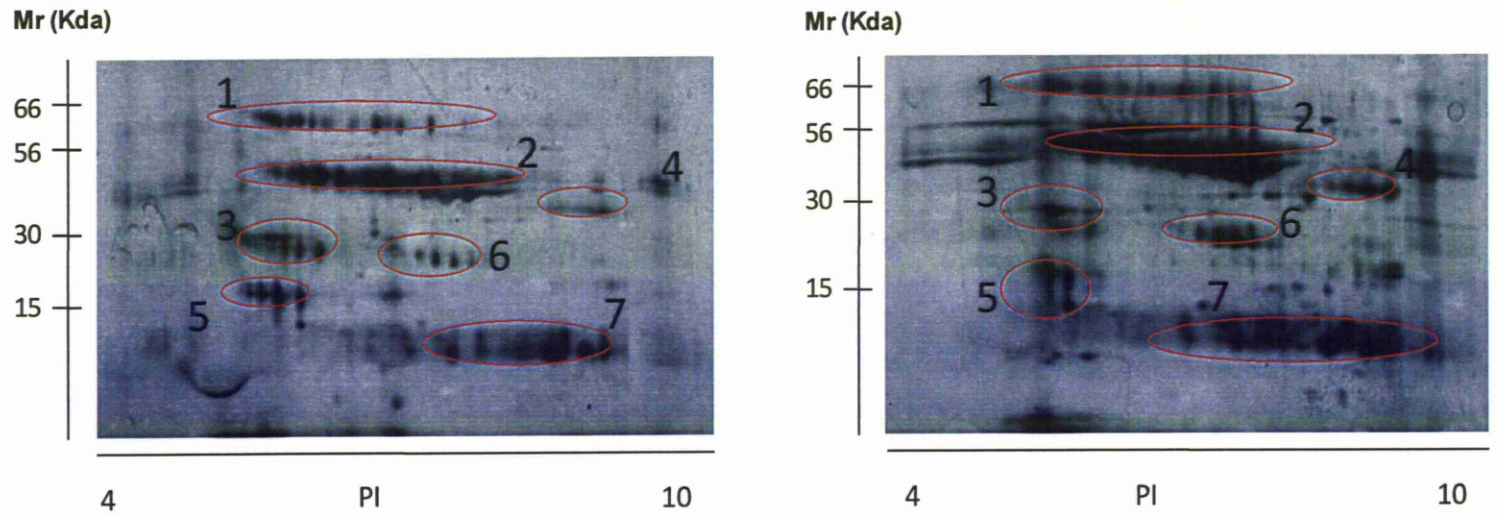

Figure 6.12 2D SDS PAGE Gels (Deutsch sample preparation) LHS Control RHS Seradate Oral Fluid. Highlighted regions

1. Albumin 2.Alpha-amylase 3.Prolactin Inducible Protein 4.Ig-Kappa Chain 5.Cystatins 6. G-3-P dehydrogenase

7. Haemoglobin Beta Chain.

A comparison between the protein preparation methods

Each of the tested preparation methods for the Seradate samples was compared against the control. Table 6.2 shows the levels of abundant proteins present as a percentage. This can also be seen in figure 6.13.

Protein	Standard	Castagnola	Deutsch
Albumin*	**10**	**-50**	**30**
Alpha-Amylase	**-5**	**-20**	**18**
Prolactin Inducible Protein	**15**	**15**	**8**
Ig-Kappa Chain*	**-10**	**-50**	**50**
Cystatins	**-40**	**5**	**18**
G3P dehydrogenase	**-60**	**-45**	**40**
Haemoglobin Beta Chain*	**5**	**-70**	**8**

Table 6.2 The percentage of proteins presented by the Seradate in comparison to the control using 3 different sample preparation methods.

A greater percentage of proteins were presented by the Seradate in comparison to the control, this included albumin (Standard 10%, Deutsch 30%), prolactin (Standard 15%, Deutsch 8%) and hemoglobin beta chain (Standard 5%, Deutsch 8%) with both standard and Deutsch method. An increase in Prolactin was also shown in Castagnola method (15%) in comparison to the control. Both the Deutsch method and Castagnola method presented a greater amount of cystatins in comparison to the control

(Castagnola 5%, Deutsch 18%) this was higher than the level presented by the standard method (- 40%).

The results show a greater level of protein presentation using the Deutsch method in comparison to the standard and Castagnola sample preparation.

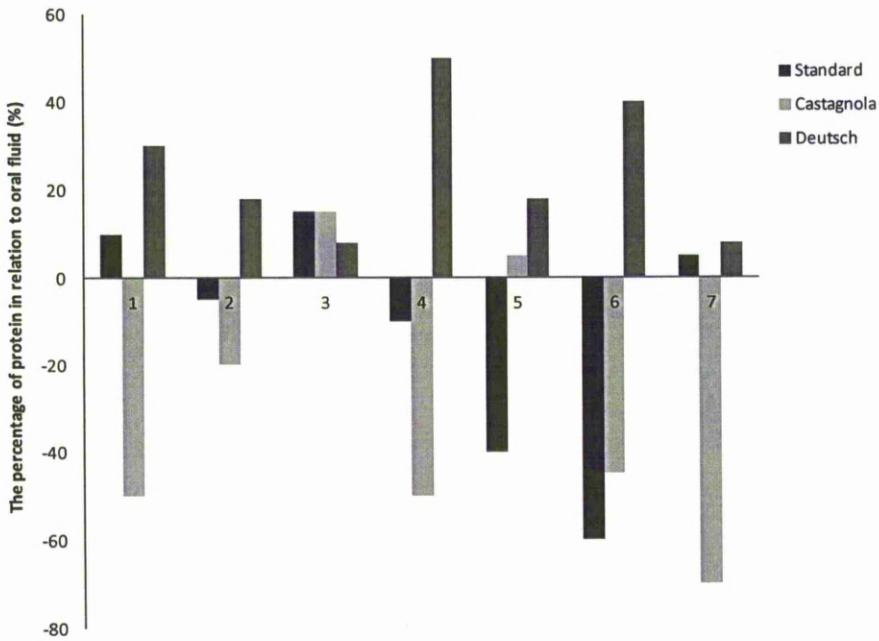

Figure 6.13 The percentage of Seradate protein Intensity in relation to control using all 3 sample preparation method. 1. Albumin 2.Alpha-amylase 3.Prolactin Inducible Protein 4.Ig-Kappa Chain 5.Cystatins 6. G-3-P dehydrogenase 7. Haemoglobin Beta Chain. The samples were pooled from 5 individuals. The SEM is too small to show graphically ≤0.5%.

6.6 Discussion

To screen for the presence of abundant proteins collected using the Seradate and OFCDs could provide potential monitoring of oral based proteins that can be identified by mass spectrometry. Our interest lay in screening abundant proteins that were presented by the previously tested OFCDs and the control (see chapter 3). For oral fluid profiling the following parameters were used.

- Oral fluid Protein profiling using 1D SDS PAGE
- Using 2D gel electrophoresis to Compare oral fluid sample preparation and its effects on protein profiling

Oral fluid Protein profiling using 1D SDS PAGE

Initial profiles were examined using 1D SDS PAGE gels, these showed comparable presentation of abundant proteins by all our tested methods. The Abundance of proteins was measured by intensity to provide a qualitative value for each of the visualized proteins. The screening of OMT derived proteins (serum-based) within the OFCDs and control included albumin, Ig-heavy and kappa chain.(Almstahl, Wikstrom et al. 2001; Ng, Koh et al. 2003; Hershkovich and Nagler 2004; Chiappin, Antonelli et al. 2007). The intensity of the protein bands of these markers was higher amongst the OFCDS in comparison to the control. Both the Oracol and Orasure (Figure 6.1) showed a greater level of protein distribution in comparison to the control, the reasons for this occurring can be related to the proteins being concentrated in a dilution dependant manner within the absorbent material; this factor is a recurring concept amongst the use of OFCD sampling

techniques, there could be also be a high protein affinity and release by the OFCDs absorbent material.(Strazdins, Meyerkort et al. 2005; Chang, Cohen et al. 2009; Kidd, Midgley et al. 2009; Kozaki, Hashiguchi et al. 2009; Almela, Hidalgo et al. 2011). The proteins banding seen within the gels could have also been caused by sample degradation during processing causing breakdown of the proteins and subsequent banding was a result of protein fragmentation (Chevalier, Hirtz et al. 2007; Schipper, Loof et al. 2007). As the data collected was qualitative, measurements of protein presence were associated to intensity rather than mass or concentration (using the mean grey area and the control sample as a reference).

Using 2D gel electrophoresis to compare oral fluid sample preparation methods and its effects on protein profiling

Both the Seradate and the control showed similar protein levels in the 1D SDS Page profile and 2D gel electrophoresis was used compare profiles of the two samples. The two samples were treated by 3 different methods of sample preparation ((Standard method (developed at the University of Liverpool, Castagnola method and Deutsch Method)) (Castagnola, Inzitari et al. 2004; Messana, Inzitari et al. 2008, Deutsch, Fleissig et al. 2008).

Our findings showed that oral fluid protein mapping using 2D gel Electrophoresis is not only dependant on the OFCD used but also on the method of sample preparation. The comparison was made between the Seradate against the control (drooled saliva). The data suggests that the use of the Deutsch method gave the most favorable results in the presentation of collected proteins using the Seradate, A greater percentage of proteins was presented by the Seradate in comparison to the control, this included albumin (Standard 10%, Deutsch 30%), prolactin (Standard 15%, Deutsch

8%) and hemoglobin beta chain (Standard 5%, Deutsch 8%) with both standard and Deutsch method and an increase in Prolactin was also shown in Castagnola method (15%) in comparison to the control.

These findings are not definitive as the results may have been affected by the staining method of used (silver staining). Silver staining is used to provide greater resolution of proteins that are mapped on a gel, there is an associated risk of over staining that can cause a shift in the final intensity of the protein spots. Further staining protocols that could be used in the future include the colloidal coomasie blue stain; fluorescent dye stains (Cy3/ Cy5). These methods will help differentiate between sample preparations used and help rule out any discrepancies caused by the silver staining method.

Closing remarks

There is a further requirement for quantitative analysis of the proteins presented by our tested OFCDs; this requires the supplementation of LC/GC mass spectrometry. Due to time constraints this was not possible, but the findings within the gels show that both Seradate and the control samples gave very similar protein profiles. The increase of the detection of OMT derived proteins by OFCDs show that there is a distinct advantage in the collection of oral fluids using a collector as opposed to testing oral fluid alone.

This provides a potential background for the uses of OFCDs in standardising protein presence in oral fluid collections, the sampling methods will allow for large scale screening studies and factors such as sample variability and low testing volumes will no longer be an issue (Messana, Inzitari et al. 2008).

Chapter 7

Discussion

Seradate is an oral fluid collection device prototype that was designed with the intention of collecting oral fluid that is OMT protein-rich. The design and concepts were based on clinical experiments conducted by Pashley et al that suggested the collection of pure OMT (GCF) from the ginigival crevice is either a serum transudate or an inflammatory exudate (Alfano 1974; Pashley 1976).

Our view was to bridge the two forms of OMT and collect a sample that would exhibit transudate/ exudate properties. The method for collecting OMT as a pure sample has been done using a variety of methods, but the most common and non-invasive methods are via the insertion of filter paper within the gingival sulcus (intra-crevicular method) or outside the gingival sulcus (extra-crevicular method) (Loe and Holmpede.P 1965; Griffiths 2003). The collected samples and their comparison to serum based proteins levels are dependent on gingival health. The collection procedure does have limitations as it is time consuming and requires a dentist, this reduces its potential applications within a non-clinical environment or for use in mass screening studies(Strazdins, Meyerkort et al. 2005).The collection procedure is non-invasive but removes the ease in sample collection that is offered by an OFCD.

Our initial aims were to create an OFCD that would potentially collect oral fluid that was richer in OMT content in comparison to current marketed collectors. This was proposed by collecting a sample by causing minor abrasion to the gum surface, the actions of rubbing (as used in the Oracol and Orasure) and mastication (our own additional function) to create an elevation in the release

of OMT while maintaining that no blood is drawn. The collector should also shield from excess saliva to reduce the dilution factor of the final collected sample.

The Seradate prototype was tested alongside current marketed OFCDs and the tested parameters included were

- Seradate design and end user acceptance
- Immunoglobulins retention and release
- Stress markers retention and release
- Measuring smoking status of individuals (cotinine levels)
- Oral fluid sample stability
- Screening of protein profiles using 1D SDS PAGE and 2D gel electrophoresis

7.1 Seradate design and end user acceptance

Seradate design

The Seradate prototype incorporated two forms of collection into its test design this included the abrasion motion and the biting motion. The dual functions of the Seradate were proposed to facilitate an increase in OMT outflow by causing mild trauma to the surfaces of the gingivae.

The absorbent material used for the collection head was D3727g; this showed the appropriate properties that were suited for our collector. The material could be easily molded and was comfortable enough to be placed within the oral cavity. It also showed a high uptake and release of tested fluids, with a low retention of testable proteins (IgG).

Seradate was commended on its material use and comfort upon placement in the mouth in particular the gum surfaces. Contraindications were that the head of the applicator was too large and a reduction in the size of the absorbent material would have overall enhanced the experience.

End user acceptance

The validation of the Seradate prototype must work in tandem with the acceptance of the collector by the end user, the questionnaire used throughout the process of this study (See Appendix A and C) was to measure individual concerns and compliance with the collector and individual input into a list of limitations that may have been overlooked, that would provide future improvement. One of the main problems the volunteers stated was the swab material fell out during the collection, this is a design error and the use of pre-moulded material would have been better suited for the collection procedure. The handle of the collector should've been more rigid and collection head should have been more flexible to allow easier placement with the oral cavity. The collection head was slightly too large and the material used (polyurethane) should've been softer especially in the collection head. The overall compliance of the Seradate for use in Oral fluid collection was acceptable by all users.

7.2 Seradate and monitoring of oral fluid componants

Immunoglobulins (IgA, IgG and IgM)

Our findings show that the Seradate collection actions of abrasion and mastication to the gum surfaces did result in an increase in the capture of our surrogate marker for OMT (IgG), this increase was marginally higher in comparison our control (drooled oral fluid). Possible reasons for this occurring are linked to low levels of trauma caused to the gum surfaces. The levels of IgG measured were comparable to all tested OFCDs with the exception of the Oracol which collected a predominantly higher amount of IgG and the Concateno Certus which collected the least.

In comparison to our collection of OMT (intra-crevicular and extra-crevicular) all tested OFCDs showed a range of IgG that was between 15-25 fold lower in measured levels.

The levels of IgA measured were significantly higher in the control and Seradate collection device, as our main purpose in the development of the Seradate was to shield from salivary secretions, adequate measures were not in place to account for the collection of saliva from the floor of the mouth where most of the saliva collected, this would account for the higher IgA content witnessed in the Seradate collector, this is an additional component that can be validated for future OFCDs in their ability to monitor salivary antibodies.

IgM is generally found in lower volumes in oral fluid due to size of the compound; the levels of IgM were marginally lower in comparison to measured IgG levels. This suggests that IgM levels are often masked by IgG. IgM provides an alternate method for validation of an oral fluid sample. As IgM is large compound, it has a greater potential to bind to the collection material. Further

validation on the release of IgM from respective OFCDs may provide insight into the use of IgM in future studies.

The IgG: IgA ratio was used as an indicator for affinity for each OFCD in its ability to gain a more OMT-rich Oral fluid sample .The ratio was comparable between all OFCDs, with the exception of Oracol. The Oracol presented the highest IgG: IgA ratio therefore highlighting its proficiency in the collection of OMT based antibodies.

The levels of Immunoglobulins collected by the Seradate were comparable to that of the control the material used in Seradate exhibited a low retention of constituents, its uses within the OFCD have been validated.(Strazdins, Meyerkort et al. 2005; Chang, Cohen et al. 2009; Kidd, Midgley et al. 2009; Kozaki, Hashiguchi et al. 2009).

The Seradate was designed to shield from the parotid gland secretions but the swab portions of the collector were exposed to sublingual and submandibular salivary secretions, caused by contact with the floor of the mouth. The increase in collected saliva directly diluted IgG content of the collected sample; this reduced the measurement of any elevation in IgG collected. As an alternative method to the collection, would have been to remove the absorbent material exposed to floor of the mouth and only process the material that is in contact with the anterior gum surface.

The order of collection of oral fluid samples was studied to measure the effects of stimulation on measured IgG levels within the tested samples. The samples were collected on 3 separate occasions to account for differences in levels monitored. Initial collection was done in the standard order, and was compared to

reverse order of collection on 2 separate occasions. The times of sample collection were kept the same to account for diurnal variability. The final data showed no significant difference in the samples collected. As each method was conducted and allowed for adequate re-establishment of the oral cavity resting state, there was no variation shown.

Cotinine

The Seradate was tested on its ability to accurately measure cotinine. The cotinine assay used was standardised for all OFCDs (dilution factors were taken into account for the Orasure and Certus).

The cotinine assay assessment for non-smokers was given as a negative value, as the values fell below the range of the assay, the male and females tested for smoking showed varying levels of cotinine by each tested collection method, in particular the Seradate and Oracol, these showed a slight significant difference in collection in comparison to the other OFCDs and control.

The material based interference of the Seradate swab would require further validation in the measurement of cotinine. As cotinine testing by ELISA is primarily used as a screening method, this finding could be inconsequential. The levels of cotinine measured in the study were relatively low (mild smoking range), comparisons to medium/ heavy smoking groups would possibly provide a more detailed assessment of all tested OFCDs.

Stress markers

The levels of albumin, alpha-amylase and cortisol were measured to establish the effects of smoking on stress markers, the results showed inconclusive data with the regard to the proposed hypothesis. The induction of stress was proposed as the samples were collected 2 weeks prior to the summer examinations of volunteers within the test group. However the levels of cotinine measured within the study didn't account for long term smokers and cotinine levels dictated a low to moderate level of cigarette consumption. A study involving a larger cohort of heavy to moderate/light smokers would have provided a greater depth to the study. As the literature on smoking and stress mainly deals with the effects of stress upon smoking cessation this provides another unique parameter by which to assess the effects of smoking on stress hormones(Benowitz, Lessov-Schlaggar et al. 2006; Badrick, Kirschbaum et al. 2007; Fu, Fernandez et al. 2009; Darlow and Lobel 2012; Hauge, Torgersen et al. 2012). The measurement of alpha-amylase activity was subjected to one test parameter, as alpha-amylase levels are affected by the flow rate of saliva, an additional test would have been to measure secretion rate of amylase also. It is stated in the literature that salivary flow on tested markers can be influenced by the sampling collection methods used (DeCaro 2008; Almela, Hidalgo et al. 2011). As we compared the ability of each OFCD to measure oral-based hormones, this additional test would provide a further validation step for the use of the Seradate and subsequent validation for other OFCDs.

7.3 Seradate and oral fluid sample stability

The effects of degradation of OFCD samples were compared to that of the control, our findings showed that samples remained stable for a shorter period of time outside of freezing temperature(-20°C)(Chevalier, Hirtz et al. 2007; Schipper, Loof et al. 2007; Almela, Hidalgo et al. 2011). It is suggested within the literature that oral fluid samples that are used in proteomic profiling are subjected to snap-freezing or immediately placed in -80°C refrigeration upon processing of the samples. This is to reduce oral fluid breakdown that is caused by salivary and bacterial proteolysis. The addition of protease inhibitors to the OFCD samples and control (with the exception of Certus and Orasure) showed reduction in IgG degradation over a period of 7 days at room temperature and 14 days at 4°C but IgG stability showed a steady decline after this point. The samples showed stability at -20°C throughout the duration of the study. The exceptions to the levels of IgG degradation were seen in the Certus and Orasure, both of which use an anti-bacterial buffer.

The stability of cotinine only showed mild signs of degradation after the two week period, this is accounted for in the literature as cotinine is no longer metabolised once the sample is collected and any form of degradation is caused by bacterial involvement. As the differences in cotinine levels were stable these samples could remain at room temperature for a period of up to 28 days. Oral fluid samples should be kept at -20°C immediately after collection if possible to prevent any breakdown, and to allow for accurate testing of oral fluid compounds.

For long term storage samples should either be snap-frozen in liquid Nitrogen or placed at -80°C refrigeration(Messana, Cabras et al. 2004; Chevalier, Hirtz et al. 2007; Schipper, Loof et al. 2007; Almela, Hidalgo et al. 2011).

7.4 Seradate and protein profiling

The protein profiling study that compared the levels of proteins presented by OFCDs against the control, showed the presence of OMT markers within each collected fluid. This alongside the 2D gel electrophoresis protein profile showed no significant difference in protein profiles between the Seradate and the control drooled oral fluid. Within the sample preparation methods the Deutsch method (Deutsch, Fleissig et al. 2008) gave a clearer profile in comparison to the Castagnola method (Messana, Inzitari et al. 2008) and standard method of sample preparation. As the study only indicated the screening of abundant proteins that were of oral fluid and OMT origin, no significant differences in profiles were seen, this is suggestive for the use of OFCDs within salivary based proteomic studies and could potentially provide a method of enhancing protein presentation for easier identification.

7.5 Overview

The tests conducted on the Seradate prototype show it is able to function comparably and favourably in the monitoring of oral fluid compounds and in comparison to tested OFCDs.

With further refinement of design and material based testing, the Seradate has the potential for being a marketable property and these factors will be taken into consideration for the next phase of development.

7.6 Conclusion

The Seradate OFCD showed promising results in its uses as an oral fluid collection device. The validation tests conducted show that its functionality is comparable to current marketed collectors.

The collection of testable proteins, drugs and hormones have shown comparable levels with normal drooled oral fluid. This shows the potential role for the Seradate in the standardisation of oral fluid for future sample testing.

7.7 Future work

The design, manufacture and testing of a unique oral fluid collection device was validated throughout this thesis.

The Seradate was designed on established collection methods and was combined with unique alterations to increase the collection of OMT-rich oral fluid.

The 35 feedback questionnaires taken throughout the study (see Appendix C) provided a background for both user acceptance and user mediated improvement on the Seradate and tested OFCDs. The feedback showed that although the Seradate was deemed acceptable by all users, it was not without limitations. These points can be used as a guideline for streamlining the Seradate and other OFCDs as potential swabs, by taking into account improvements and limitations within each tested OFCD.

The further development and validation of the Seradate include

- Design
- Material testing
- Selective measurements of oral fluid components
- Oral fluid protein profiling
- Marker detection assay and lateral flow immunochromatographic assays

These factors can be used to improve on and establish further research into the development of an OFCD.

Design

The limitations to the design of the Seradate were related to its unique method of collection (a combination of mastication and abrasion). Our findings showed that the mild abrasion caused by the Seradate caused a marginal increase in the levels of OMT released in comparison to the marketed OFCDs. As OMT release is dictated by gum health, this method alone will be insufficient in normal individuals. Our study on the use of filter paper to acquire OMT samples showed an elevation in OMT constituents (IgG), this method is well established and a method for standardising this collection needs to be addressed.

Material tests

The material tests for the seradate used the polyolefin material as it satisfied the criteria for our collection purposes. As the method of abrasion and mastication used only gave comparable levels of oral based constituents to OFCDs, a proposal for the use of sintered plastics can be suggested. The use of sintered plastics could be used in a similar manner as the pure OMT collection method stated in the thesis. This method would involve placement of the sintered plastic against the area of interest and removed once the material is saturated. With direct contact to the gum surface, OMT will be collected by capillary attraction. This method can incorporate shielding to reduce dilution of the final collected sample by saliva.

Selective tests on oral compounds

The tested parameters for the validation of the Seradate looked at OMT specific components such as serum based immunoglobulins IgG, IgM, Albumin and salivary based IgA, alpha-amylase, cortisol. To test the accurate measurements for drugs found in oral fluid, the levels of cotinine were measured.

These tests provide a base from which further studies can be conducted, our findings with Seradate show that the collector in its current form is comparable to marketed OFCDs. By increasing the range of testable OMT/ Oral fluid components this will provide a stronger foundation for the acceptability of oral fluid for screening purposes and will aid in the standardisation of oral fluid.

Potential avenues that can be looked at include

- The measurement of cancer markers presented in oral fluid
- Diabetes and cardiovascular markers
- Screening of immunoglobulins associated with viral disease

Oral fluid protein profiling

The study on protein profiling of oral fluid provided an answer to the standardisation of a generic oral fluid sample (a whole saliva sample). Current research in oral fluid has shown that inter and intra-variability of sample collection causes problems to standardisation. As protein profiling of OMT and oral fluid have shown slight variance in composition this requires further validation and with 2d- gel electrophoresis coupled with mass spectrometry this can be developed further. To limit changes caused gel imaging and staining more appropriate methods of

detection such as DIGE (differential gel electrophoresis) can be used, where two samples are run on the same gel and each sample is tagged with a unique spectrally resolvable florescent dye (Cy2,Cy3 and Cy5).

As the proteome of oral fluid is being analysed for its individual and collective secretions this can provide more robust information for potential markers associated with localised (oral) and systemic diseases.

Marker detection assay and lateral flow immunochromatographic assays

As the proposed use of Seradate is to provide fast and easy methods of oral fluid collection for screening purposes, this can be supplemented further with the development and standardisation of serum based immunoassay to account oral fluid based test components.

Lateral flow immunochromatographic assays provide a potential gap in the market especially where laboratory facilities are reduced and for on-site validation. This method can be developed to provide screening services for cancer markers presented in oral fluid (local and systemic), localised disease (periodontitis) systemic disease markers (diabetes, cardiovascular) and immunoglobulins associated with viral disease.

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Appendix A

- ***Informed Consent***
- ***Feedback Questionnaire***
- ***Information Sheets***
- ***Recruitment advertisement***
- ***Ethics Documentation***

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INFORMED CONSENT FORM

Title of Research Project:	A comparison of different oral swabbing methods in the collection of whole saliva
Researcher(s):	Mr. Abadur Rohman, Dr.Stuart Marshall-Clarke

Please initial box

1. I confirm that I have read and have understood the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my rights being affected. ☐
3. I understand that, under the Data Protection Act, I can at any time ask for access to the information I provide and I can also request the destruction of that information if I wish. ☐
4. I agree to take part in the above study. ☐

Participant Name	Date	Signature
Name of Person taking consent	Date	Signature
Researcher	Date	Signature

The contact details of lead Researcher (Principal Investigator) are:

Dr. Stuart Marshall-Clarke
Institute of Translational medicine
Telephone: (0151) 794 5444
Email: stumc@liv.ac.uk

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An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number
Smoking Status

Please tick appropriate box:

Dental visits	Regular	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience
Method 1				
Method 2				
Method 3				
Method 4				
Method 5				
Method 6				
Method 7	NULL			

Further Comments (please continue overleaf if required)

UNIVERSITY OF LIVERPOOL

Information Sheet

A comparison of different oral swabbing methods in the collection of whole saliva

You are invited to participate in an exciting area of research. Prior to deciding whether to participate, it is important that you understand why the research is being conducted and also what it will involve. Please take time to read the following information carefully and if you're unclear with any terms or anything you don't understand feel free to ask us. You are also at complete liberty to discuss this with family, friends or G.P if you wish. We would like to highlight at this point that you are under no obligation to continue with the testing if you wish to withdraw at anytime and you should only agree to continue if you want to.

What is the Purpose of study?

There are currently various whole saliva swabs on the market. In this study we aim to assess different swabbing methods alongside a swab we developed to compare their overall ability in collecting whole saliva and to indicate smoking status.

Why have I been chosen to take part?

We are looking for healthy individuals in the university to test our oral swab alongside current marketed oral swabs. We are looking for smokers and non-smokers between the ages of 18-30. The swabs will be tested for smoking status.

Do I have to take part?

Your participation within the study is completely voluntary and you are free to withdraw from the study at anytime without explanation or incurring any disadvantage.

What will happen if I take part?

You will be asked to give 7 whole saliva samples using different swabbing methods. The procedures will be monitored by a dentist and a suitably trained individual.

Collection Procedure

The estimated total time for collection of all samples will be between 1 and 2 hours. The collection will take place in groups of 5 (Individual screening will also be available).

You will be asked to return on one further occassion to provide a second series of samples at a later date in the week. The estimated collection time will be 1 hour.

The times for collection will be given to you. All procedures will be monitored by a suitably trained individual.

Questionaire

Upon completion you will be asked to complete an informal questionaire. The informal questionaire will ask you to evaluate your experience of the ease of use and user friendliness for each of the swabs.

The results from the study will be made available to you upon request after completion of the study. You will be in the presence of research staff throughout all procedures. Please feel free to ask any questions if you are unsure at any stage.

Expenses and Payments

Upon completion of your participation you will receive re-imbursement for your time.

Are there any risks in taking part?

There are no risks involved. However if you feel discomfort at anytime. Please report this to research staff immediately.

What If I'm unhappy or if there is a problem?

If you are unhappy at any stage during the study please contact the Principal investigator *Dr. Stuart Marshall-Clarke* (0151) 794 5444 (stumc@liv.ac.uk) and we will try to help. If however your complaint remains unresolved and you feel you cannot talk to us you should contact the Research Governance Officer on 0151 794 8290 (ethics@liv.ac.uk). When contacting the Research Governance Officer please provide your name and description of the study conducted (so it can be identified), the researcher(s) involved, and the details of complaint which you wish to make.

Will my participation be kept confidential?

All data collected will be anonymised and stored securely by the Custodian Dr. Stuart Marshall-Clarke (principal Investigator).The tests conducted are not appropriate for assesment of health status and therefore no information will be provided on participants' health as part of the study.

Will my taking part be covered by an insurance scheme?

Participants taking part in a University of Liverpool ethically approved study will have cover.

What will happen if I want to stop taking part?

You are free to withdraw from the study at anytime without explanation. Any results we gain prior to this point may be used, if you are happy with this. Otherwise they will be destroyed and no further use will be made of them.

Who can I contact if I have further questions?

If you have any further enquiries please contact ***Dr. Stuart Marshall-Clarke*** (0151) 794 5444 (stumc@liv.ac.uk)

Recruitment advertisement

Institute of Translational Medicine

A comparison of different oral swabbing methods in the collection of whole saliva

We are looking for 20 healthy volunteers (smokers and non-smokers) to provide oral swab saliva samples , to compare differences in oral swabbing methods. Participants will be reimbursed for their time.

If you are interested please contact Abadur Rohman (abadur.rohman@gmail.com) or 07828676918

Version 3 18/03/2012

UNIVERSITY OF LIVERPOOL

COMMITTEE ON RESEARCH ETHICS

APPLICATION FOR APPROVAL OF A PROJECT INVOLVING HUMAN PARTICIPANTS, HUMAN DATA, OR HUMAN MATERIAL

This application form is to be used by researchers seeking approval from the **University Committee on Research Ethics** or from an approved **School Research Ethics Committee**.

Applications to the University Research Ethics Sub-Committees, with the specified attachments, should be **submitted electronically to** ethics@liv.ac.uk. Applications to an approved School / Departmental Committee should be submitted to their local address, available at http://www.liv.ac.uk/researchethics/deptcommittees.htm.

RESEARCH MUST NOT BEGIN UNTIL ETHICAL APPROVAL HAS BEEN OBTAINED

This form must be completed by following the guidance notes, accessible at www.liv.ac.uk/researchethics.

Please complete every section, using N/A if appropriate.

Incomplete forms will be returned to the applicant.

BEFORE COMPLETING YOUR APPLICATION PLEASE CONFIRM WHAT APPROVAL YOU ARE SEEKING (please check):

a) Expedited review of an individual research project ☐
b) Full committee review of an individual research project ☒
c) Expedited generic* approval ☐
d) Committee review generic* approval ☐

*to cover a cohort of projects using similar methodologies. Boundaries of the research must be defined clearly. Approval may be granted for up to 5 years and will be subject to annual review.

Office Use Only *(for final hard copies)*

Reference Number: RETH

Date final copy received:

Approval decision:

Approved – no conditions	☐
Committee	☐
Chairs Action	☐
Expedited	☐
Approved with conditions	☐
Committee	☐
Chairs Action	☐
Expedited	☐

Declaration of the:

Principal Investigator ☐ OR Supervisor and Student Investigator ☒
(please check as appropriate)

- The information in this form is accurate to the best of my knowledge and belief, and I take full responsibility for it.
- I have read and understand the University's Policy on Research Ethics
- I undertake to abide by the ethical principles underlying the Declaration of Helsinki and the University's good practice guidelines on the proper conduct of research, together with the codes of practice laid down by any relevant professional or learned society.
- If the research is approved, I undertake to adhere to the study plan, the terms of the full application of which the REC has given a favourable opinion, and any conditions set out by the REC in giving its favourable opinion.
- I undertake to seek an ethical opinion from the REC before implementing substantial amendments to the study plan or to the terms of the full application of which the REC has given a favourable opinion.
- I understand that I am responsible for monitoring the research at all times.
- If there are any serious adverse events, I understand that I am responsible for immediately stopping the research and alerting the Research Ethics Committee within 24 hours of the occurrence, via ethics@liv.ac.uk.
- I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to security and confidentiality of personal data.
- I understand that research records/data may be subject to inspection for audit purposes if required in future.
- I understand that personal data about me as a researcher in this application will be held by the University and that this will be managed according to the principles established in the Data Protection Act.
- I understand that the information contained in this application, any supporting documentation and all correspondence with the Research Ethics Committee relating to the application, will be subject to the provisions of the Freedom of Information Acts. The information may be disclosed in response to requests made under the Acts except where statutory exemptions apply.
- I understand that all conditions apply to any co-applicants and researchers involved in the study, and that it is my responsibility to ensure that they abide by them.
- **For Supervisors:** I understand my responsibilities as supervisor, and will ensure, to the best of my abilities, that the student investigator abides by the University's Policy on Research Ethics at all times.
- **For the Student Investigator:** I understand my responsibilities to work within a set of safety, ethical and other guidelines as agreed in advance with my supervisor and understand that I must comply with the University's regulations and any other applicable code of ethics at all times.

Signature of Principal Investigator ☐ or Supervisor ☒: .....
Date: (18/03/2012)
Print Name: Dr. Stuart Marshall-Clarke

Signature of Student Investigator: .....
Date: (18/03/2012)

Print Name:

SECTION A - IDENTIFYING INFORMATION

A1) Title of the research (PLEASE INCLUDE A SHORT LAY TITLE IN BRACKETS).

A comparison of different oral swabbing methods in the collection of whole saliva

A2) Principal Investigator ☐ **OR Supervisor** ☒ **(please check as appropriate)**

Title:	Dr	**Staff number:**	
Forename/Initials:	Stuart	**Surname:**	Marshall-Clarke
Post:	Senior Lecturer	**Department:**	Institute of translational medicine
Telephone:	0151 794 5444	**E-mail:**	stumc@liv.ac.uk

A3) Co-applicants (including student investigators)

Title and Name	Post / Current programme (if student investigator)	Department/ School/Institution if not UoL	Phone	Email
Mr. Abadur Rohman	PhD Student	Institute of translational medicine	07828676918	abadur.rohman@gmail.com

SECTION B - PROJECT DETAILS

B1) Proposed study dates and duration (RESEARCH **MUST NOT BEGIN** UNTIL ETHICAL APPROVAL HAS BEEN OBTAINED)

Please complete as appropriate:

EITHER

a) Starting as soon as ethical approval has been obtained ☒ (please check if applicable)

Approximate end date:	01/08/2012

OR

b) Approximate dates:

Start date:		**End date:**	

B2) Give a full lay summary of the purpose, design and methodology of the planned research.

Background
Recent attention in medical screening has turned to whole saliva as means of diagnosis for disease, drugs of abuse and smoking status.
The constituents of whole saliva include saliva secretions from minor and major salivary glands and serum-like oral mucosal transudate. Oral mucosal transudate (OMT) is released as a flushing mechanism within the gum lining to prevent infection. This fluid exhibits serum like properties and this makes it of great interest because it offers the potential to screen for serum based markers non-invasively using whole saliva samples. IgG is a consituent of serum so its quantification in whole salive samples affords a means of assessing the contribution of OMT to the sample.

Collection of whole saliva provides rich in OMT could provide a rapid patient-friendly means of monitoring disease process, drugs of abuse or smoking status (by measuring a constituent of nicotine known as cotinine).

Currently a number of devices are available for collecting whole saliva this is done either by a swab or a drooled saliva sample. The method of swabbing involves either rubbing or placing the swab near the gums until fully saturated. Very little work has been done to assess or optimise these devices for their performance in collecting OMT rich saliva.

Aims

We have recently developed a novel device for collection of whole saliva. It has been specifically designed to maximise collection of OMT-rich samples. The aim of this study is to compare our device with those currently available commercially. To assess device effectiveness we will compare the volume of whole saliva collected and assess the OMT content of the samples by measuring IgG levels. To assess the usefulness of the device in a likely application we will also compare its ability to detect cotinine in samples from smokers and non-smokers. For a full assessment of the extent of OMT contribution to the samples obtained we will also collect "pure" OMT from the gingival crevice.

We will also assess the ease and comfort of use by asking each study subject to complete a short questionaire.The purpose of the questionaire is to gauge user friendliness of the collection methods tested. The questionnaire will ask for the volunteers smoking status and maintenance of oral hygiene. It will also ask for preferences in the swabbing methods tested. The factors that will be included in the questionaire are the aesthetics of the swab, ease of collection, comfort in use and overall experience. This will be graded on a 1 to 10 scale (1= Very Poor; 10= Excellent). The volunteers will be free to add any further comments they want to share with regard to the study.

Cohort

For this study we require 20 individuals male/female (10 smokers and 10 non-smokers) recruited from the University of Liverpool (age group of 18-30). Volunteers will be recruit by advertising the study on the University Announcements page and written consent will be sort prior to engaging with the collection of samples.The results from the tests will be available to individuals upon request. All data collected will be stored in accordance Data protection act 1998 and Freedom of information act (2000)

Methods of oral swabbing

Method 1

This method involves the placement of a filter paper strip into the gingival crevice (the gap between gum and tooth) for a period of 30 seconds. This procedure is well established and will be carried out by a qualified, experienced dentist.

Method 2

Our developed swab (Seradate) simply requires the participant to 'bite' down on a swab for two 30 second periods with a brief rest in between.

Method 3

The Orasure® swab requires the volunteer to swab the lower cheek and gum, until the pad is moist. The pad is held in place for a period of 2 minutes after which it will be taken out.

Method 4

The Salivette® swab is placed either above or below the tongue. Once the material is saturated it is removed.

Method 5

The Certus® swab is used in the same manner as the Orasure®.

Method 6

The Oracol® swab is used in the same manner as the Orasure

Method 7

Passive Drooled whole saliva, the collection is done by askin volunteers to passively drool into a tube.

Sample Storage

Each of the collected samples will be placed in preservative and placed in -80'C freezer until required for testing. The Storage of the samples and work will be conducted in the university premesis (Licensed) and will be done in accordance with the Human Tissue Act.

Collection Procedure

Primary Collection

Each procedure will take 5 minutes and will be followed by a rest period before the next procedure is undertaken. Volunteers will be asked rinse their mouths with cold water between each swabs.
The estimated total time for collection of all samples will be between 1 and 2 hours. The collection will take place in groups of 5.

Repeat Collection
A second series of samples will be collected within the following 7 days using only methods 2-7.The estimated time for collection will be 1 hour.This is will be done so as multiple samples are available for testing and will help address any inconsistencies in the measured markers (IgG and Cotinine) within the study,as well as assess the bias in order of collection by the swabs..

B3) List any research assistants, sub-contractors or other staff not named above who will be involved in the research and detail their involvement.

Dr. Bhavish Patel (Collection of sample 1)

B4) List below all research sites, and their Lead Investigators, to be included in this study.

Research Site	Individual Responsible	Position and contact details
Institute of translational medicine	Abadur Rohman	PhD Student 07828676918

B5) Are the results of the study to be disseminated in the public domain?

YES ☒ NO ☐

- ***If not, why not?***

B6) **Give details of the funding of the research, including funding organisation(s), amount applied for or secured, duration, and UOL reference**

Funding Body	Amount	Duration	UoL Reference
Self funded	£2500	1 year	

B7) **Give details of any interests, commercial or otherwise, you or your co-applicants have in the funding body.**

none

SECTION C - EXPEDITED REVIEW

C1)

a) Will the study involve recruitment of participants outside the UK?	No
b) Does the study involve participants who are particularly vulnerable or unable to give informed consent? *(e.g. children, people with learning or communication disabilities, people in custody, people engaged in illegal activities such as drug-taking, your own students in an educational capacity) (Note: this does not include secondary data authorised for release by the data collector for research purposes.)*	No
c) Will the study require obtaining consent from a "research participant advocate" (for definition see guidance notes) in lieu of participants who are unable to give informed consent? *(e.g. for research involving children or, people with learning or communication disabilities)*	No
d) Will it be necessary for participants, whose consent to participate in the study will be required, to take part without their knowledge at the time? *(e.g. covert observation using photography or video recording)*	No
e) Does the study involve deliberately misleading the participants?	No
f) Will the study require discussion of sensitive topics that may cause distress or embarrassment to the participant or potential risk of disclosure to the researcher of criminal activity or child protection issues? *(e.g. sexual activity, criminal activity)*	No
g) Are drugs, placebos or other substances (e.g. food substances, vitamins) to be administered to the study participants or will the study involve invasive, intrusive or potentially harmful procedures of any kind?	No
h) Will samples (e.g. blood, DNA, tissue) be obtained from participants?	Yes
i) Is pain or more than mild discomfort likely to result from the study?	No
j) Could the study induce psychological stress or anxiety or cause harm or negative consequences beyond the risks encountered in normal life?	No
k) Will the study involve prolonged or repetitive testing?	Yes

l) Will financial inducements (other than reasonable expenses and compensation for time) be offered to participants?	No

C2)

a) Will the study seek written, informed consent?	Yes
b) Will participants be informed that their participation is voluntary?	Yes
c) Will participants be informed that they are free to withdraw at any time?	Yes
d) Will participants be informed of aspects relevant to their continued participation in the study?	Yes
e) Will participants' data remain confidential?	Yes
f) Will participants be debriefed?	Yes

If you have answered 'no' to all items in SECTION C1 and 'yes' to all questions in SECTION C2 the application will be processed through expedited review.

If you have answered "Yes" to one or more questions in Section C1, or "No" to one or more questions in Section C2, but wish to apply for expedited review, please make the case below. See research ethics website for an example "case for expedited review".

C3) **Case for Expedited Review** – *To be used if asking for expedited review despite answering YES to questions in C1 or NO to answers in C2.*

The level of potential risk of harm to subjects is minimal as the collection methods described are oral swabbing techniques. The subjects will be monitored by approved staff at all times.

SECTION D - PARTICIPANT DETAILS

D1) **How many participants will be recruited?**

20

D2) **How was the number of participants decided upon?**

The number was decided to account for number of swabbing methods tested. This will also lower chances of sample variability caused by collecting from a smaller cohort.

D3)

a) **Describe how potential participants in the study will be identified, approached and recruited.**

Invitation for participation in the study will be posted on the University network, Sydney Jones Library, Harold Cohen Library and University Guild of students.

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b) **Inclusion criteria:**

The inclusion criteria will be healthy volunteers aged 18-30. All volunteers will be assessed by The student (Abadur Rohman) for normal oral health (Primary teeth should be white and opaque with smooth surfaces on front teeth and grooved surfaces for back or posterior teeth, Permanent teeth should appear creamier in color and larger than primary teeth, Lips and tongue should be soft, pink, and moist tissues under the lip should appear pink or brown (depending on skin colour). The palate or upper groove of the mouth should be soft, pink and moist and skins and tissues of the face should not be bruised, swollen, or tender).A second opinion would be sought from Dr. Bhavish Patel if conclusions are not accurately made. Any issues regarding oral health can be sought from Dr. Bhavish Patel.

c) **Exclusion criteria:**

Exclusion criteria will be assesed on oral health of individuals by the student (Abadur Rohman) and Dr. Bhavish Patel.

d) **Are any specific groups to be excluded from this study? If so please list them and explain why:**

No individuals will be excluded with regards to ethnicity/gender.

e) **Give details for cases and controls separately if appropriate:**

N/A

f) **Give details of any advertisements:**

Invitation for participation in the study will be posted on the University network, Sydney Jones Library, Harold Cohen Library and University Guild of students.

D4)

a) **State the numbers of participants from any of the following vulnerable groups and justify their inclusion**

Children under 16 years of age:	0
Adults with learning disabilities:	0
Adults with dementia:	0
Prisoners:	0
Young Offenders:	0
Adults who are unable to consent for themselves:	0
Those who could be considered to have a particularly dependent relationship with the investigator, e.g. those in care homes, students of the PI or Co-applicants:	0
Other vulnerable groups (please list):	0

b) **State the numbers of healthy volunteer participants:**

Healthy Volunteers	**20**

D5)

a) **Describe the arrangements for gaining informed consent from the research participants.**

Information sheets and consent forms will be available both in electronic and paper form.

b) **If participants are to be recruited from any of the potentially vulnerable groups listed above, give details of extra steps taken to assure their protection, including arrangements to obtain consent from a legal, political or other appropriate representative in addition to the consent of the participant** *(e.g. HM Prison Service for research with young offenders, Head Teachers for research with children etc.).*

N/A

c) **If participants might not adequately understand verbal explanations or written information given in English, describe the arrangements for those participants (e.g. translation, use of interpreters etc.)**

Arrangements will be made to help translate the documentation in various languages and where needed involvement of interpreters will be sort.

d) **Where informed consent is not to be obtained (including the deception of participants) please explain why.**

N/A

D6) **What is the potential for benefit to research participants, if any?**

The Benefits for the research are to assess the different whole saliva swabbing methods and how effective they are collecting whole saliva for subsequent testing.

D7) **State any fees, reimbursements for time and inconvenience, or other forms of compensation that individual research participants may receive. Include direct payments, reimbursement of expenses or any other benefits of taking part in the research?**

Fees for time and inconvieniance caused during the study will be via direct payment of £10 per individual.

SECTION E - RISKS AND THEIR MANAGEMENT

E1) **Describe in detail the potential physical or psychological adverse effects, risks or hazards (minimal, moderate, high or severe) of involvement in the research for research participants.**

The risks associated with the procedure are minimal. There are no adverse effects associated that are either physical or psychological. The procedure detailed previously will involve natural biting mechanism, any subsequent discomfort will be addressed. The procedures implemented express negligible adverse effects.

E2) **Explain how the potential benefits of the research outweigh any risks to the participants.**

The potential benefits of the research outweigh the risks, as the methods used involve minimal risk. The potential benefits of conducting the research illustrate the promotion of whole saliva and its implications to be used as effective diagnostic medium for the assessment and treatment of disease states.

E3) **Describe in detail the potential adverse effects, risks or hazards (minimal, moderate, high or severe) of involvement in the research for the researchers.**

The risk and hazards to the researchers are minimal. The policies directed in the university code of research practice will be adhered to strictly.Both informed consent and safe tissue disposal in accordance to the human tissue act will be adhered to.All Samples will be placed in to preservative post collection to prevent bacterial growth.All Samples will be handled in a class II cabinet during testing.

E4) **Will individual or group interviews/questionnaires discuss any topics or issues that might be sensitive, embarrassing or upsetting, or is it possible that criminal or other disclosures requiring action could take place during the study (e.g. during interviews/group discussions, or use of screening tests for drugs)?**

YES ☐ NO ☒

- ***If Yes, give details of procedures in place to deal with these issues.***

E5) **Describe the measures in place in the event of any unexpected outcomes or adverse events to participants arising from their involvement in the project**

The measures in place will adhere to university of Liverpool regulations of reporting serious adverse events. These will be stipulated on both consent forms and questionnaires. The research will be halted by the principal investigator and the event reported and logged to the CORE, Sub-committee or departmental ethics board (research governance officer within 24 hours of the incidence).

E6) **Explain how the conduct of the project will be monitored to ensure that it conforms with the study plan and relevant University policies and guidance.**

The research will be monitored using the university code of research practice.

SECTION F - DATA ACCESS AND STORAGE

F1) Where the research involves any of the following activities at any stage (including identification of potential research participants), state what measures have been put in place to ensure confidentiality of personal data (e.g. encryption or other anonymisation procedures will be used)

Electronic transfer of data by magnetic or optical media, e-mail or computer networks	Anonymisation
Sharing of data with other organisations	N/A
Export of data outside the European Union	N/A
Use of personal addresses, postcodes, faxes, e-mails or telephone numbers	N/A
Publication of direct quotations from respondents	N/A
Publication of data that might allow identification of individuals	N/A
Use of audio/visual recording devices	N/A
Storage of personal data on any of the following:	
Manual files	N/A
Home or other personal computers	N/A
University computers	Anonymisation/Encryption
Private company computers	N/A
Laptop computers	N/A

F2) Who will have control of and act as the custodian for the data generated by the study?

The custodian for all data generated will be both the principal investigator Dr. Stuart Marshall-Clarke.

F3) Who will have access to the data generated by the study?

Access to the data will be available to Dr. Stuart Marshall-Clarke, Abadur Rohman.

F4) For how long will data from the study be stored?

Data and tissues will be stored in compliance with the human tissue act.

SECTION G – PEER REVIEW

G1)

a) Has the project undergone peer review?

YES ☐ NO ☒

b) If yes, by whom was this carried out? (please enclose evidence if available)

SECTION G - CHECKLIST OF ENCLOSURES

Study Plan / Protocol	Yes
Recruitment advertisement	Yes
Participant information sheet	Yes
Participant Consent form	Yes
Research Participant Advocate Consent form	Yes
Evidence of external approvals	N/A
Questionnaires on sensitive topics	N/A
Interview schedule	N/A
Debriefing material	N/A
Other (please specify)	N/A
Evidence of peer review (If G1 = Yes)	N/A

Appendix B

Materials

Chemicals and Biological compounds

All chemicals and Biological compounds used were of analytical grade. Purchased from Sigma Aldrich (UK).

Elisa Test Kits

Human IgG Elisa Quantitation Kit Bethyl Laboratories Inc (Montgomery, USA)

Human IgA Elisa quantitation Kit Bethyl Laboratories Inc (Montgomery, USA)

Human IgM Elisa quantitation Kit Bethyl Laboratories Inc (Montgomery, USA)

Human albumin Elisa quantitation kit Bethyl Laboratories Inc (Montgomery, USA)

Salivary alpha amylase detection kit (Salimetrics Europe, Suffolk,UK),

Human salivary cortisol detection kit (Salimetrics Europe, Suffolk,UK),

Human Salivary cotinine detection kit (Salimetrics Europe, Suffolk,UK).

Oral fluid collection materials

All biomaterials tested and utilised for the Seradate were acquired from

Filtrona Inc (Filtrona Fibertec GmbH,Reinbeck, Germany)

Porex Technologies (Porex Technologies GmbH, Aachen, Germany)

Oral fluid collection devices

Prototype collection Device Seradate

Seradate is an oral fluid swab prototype (University of Liverpool). It is made of two hydrophilic material surfaces supplied by Filtrona Fibertec. (Germany).

Orasure

Orasure oral fluid collection device (Orasure Technologies Inc, Bethlehem, Pennsylvania) is an absorbent cotton pad affixed to a nylon stick. The pad was treated with a buffered salt solution (0.1% gelatine, 3.5% sodium chloride, 0.3% citric acid, 0.1% potassium sorbate, 0.1% sodium benzoate pH 7.2). It has its own preservative solution (0.8 ml aqueous antimicrobial preservative solution 0.5% Tween-20, 0.01% chlorohexidine digluconate)

Salivette

Salivette saliva collector (Sarstedt Numbrecht, Germany) is a plain cylindrical cotton swab designed specifically to collect saliva.

Oracol

Oracol oral fluid collector (Malvern Medical Developments, Worcester, UK) is a hydrophilic foam pad affixed to a nylon handle). It targets a mixture of OMT and saliva.

Concateno Certus

Certus oral fluid collector (Concateno global drug testing services, London UK) is made of a hydrophilic cylindrical polymer affixed to a ergonomic handle with incorporated volume adequacy indicator (white to blue once sufficient fluid has been collected ≤1ml)

Solution and Buffers

For Human IgG, IgA, IgM Elisa Assay

Phosphate Buffered Saline X10

	1L
NaCl	80g
KCl	2g
Na_2HPO_4	14.4g
KH_2PO_4	2.4g

Coating Buffer

0.05 M Carbonate-Bicarbonate, pH 9.6, dissolved capsule in 100ml of distilled water

Wash Solution

Phosphate buffered saline, 0.05% Tween 20, pH 8.0, 10X PBS (1:10 dilution of Stock) and 500µl of Tween 20. Final volume 1L.

Blocking (postcoat) Solution

Phosphate buffered saline, 1% BSA, pH 8.0, PBS (1:10 dilution of Stock) 10g BSA. Final volume 1 L

Sample/Conjugate Diluent

Phosphate buffered saline 1% BSA, 0.05% Tween 20, pH 8.0, PBS (1:10 dilution of Stock) 10g BSA, 500ul of Tween 20. Final volume 1 L

Enzyme Substrate

Tetra-methyl benzidine (TMB). Use Neat as directed by manufacturer's instruction.

Stopping Solution

4M H_2SO_4 (1 part H_2SO_4:4 parts H20)

Calibrator for standard curve

Human reference serum amount: 0.1 ml Concentration

Antibodies used in the testing of OFCDS

	Coating antibody	Concentration	Dilution from neat	HRP Detection Antibody	Concentration	Dilution from neat
IgA	Goat anti-Human IgM-affinity purified	1 mg/ml	1:100	Goat anti-Human IgM-HRP conjugate	1 mg/ml	1:100
IgM	Goat anti-Human IgM-affinity purified	1 mg/ml	1:100	Goat anti-Human IgM-HRP conjugate	1 mg/ml	1:100
IgG	Goat anti-Human IgM-affinity purified	1 mg/ml	1:100	Goat anti-Human IgM-HRP conjugate	1 mg/ml	1:100
Albumin	Affinity Purified Human Albumin coating antibody	1mg/ml	1:100	HRP Conjugated Human Albumin Detection Antibody	1 mg/ml	1:100
Cortisol	Monoclonal anti-cortisol antibodies	Unknown	N/A	HRP Conjugated cortisol antibody	Unknown	N/A
cotinine	Rabbit anti-cotinine	Unknown	N/A	HRP conjugated cotinine	Unknown	N/A

2.3 Buffers for 2D gels

Buffers

Running Buffer (10x)

Add 800ml of distilled water into a 2 litre container. Add 29g Tris base, 144g glycine and 10g of SDS. Total volume 1 litre with distilled water. Stir solution using a magnetic stirrer.

Running Buffer (1X)

Add 100ml of 10X running buffer to 900ml distilled water.

Resolving Gel Buffer (3M Tris-HCL at pH 8.85)

Add 36.3g tris base to 50ml distilled water, stirred until dissolved. pH to 8.85 using concentrated HCl. Make up total volume to 100ml with distilled water.

Stacking Gel Buffer (1.25M Tris-HCL at pH 6.8)

Add 15.1g tris base to 50ml of distilled water, stir solution using a magnetic stirrer. pH to 6.8 with concentrated HCl. Make up the Total volume to 100ml with distilled water.

SDS 10%

Add 10g of SDS to 50ml of distilled water, total volume to 100ml with distilled water, solution is stirred by magnetic stirrer.

APS

Add 100mg of APS in 1ml distilled water.

Buffers for IEF

IEF Rehydration Solution (8M Urea 2M Thio urea)

4% Chaps,12g Urea, 3.8g of thiourea and 1g of Chaps. Total volume to 25ml. Rotate until fully dissolved, add 2 or 3 grains of bromophenol blue. Store at -20°C.

Equilibration Buffer

43.47g of glycerol, 4ml of stacking gel buffer (1.25M Tris-HCl @ pH 6.8), 36.04g of urea and 2g of SDS. Make volume up to 100ml. When dissolved add 2 or 3 grains of bromophenol blue. Store at -20°C

Agarose Sealing Solution

Add 1g of agarose to 100ml of Running Buffer (1X), heat at 100°C until dissolved. Add 2 or 3 grains of bromophenol blue and store at room temperature (25°C)

Silver staining solutions

Fixing solution
25ml acetic acid, 100ml methanol, 125ml distilled water

Sensitization solution
30ml methanol, 1.0g sodium thiosulphate, 6.8g sodium acetate, 66ml distilled water

Silver stain solution
0.25g silver nitrate, 100 ml distilled water

Development solution
2.50g sodium carbonate 100ml distilled water 40ul formaldehyde

Stop solution

1.46g EDTA, 100ml distilled water

Buffers for 1D page

5x Sample Buffer

10% w/v SDS 10nM Dithiothreitol 20% v/v Glycerol 0.2M Tris-HCL 0.05%w/v bromophenol.

Running Buffer (10x)

To 1000ml of distilled water add 29g tris base, 144g glycine and 10g of SDS. Stir until dissolved via magnetic stirrer.

Running Buffer (1X)

100ml of 10X running buffer to 900ml distilled water.

Resolving Gel Buffer (3M Tris-HCL pH 8.85)

To 100ml distilled water add 36.3g tris base, stir until dissolved. pH to 8.85 with concentrated HCL.

Stacking Gel Buffer (1.25M Tris-HCL pH 6.8)

To 100ml of distilled water add 15.1g tris base, stir until dissolved. pH to 6.8 with concentrated HCL.

SDS 10%

To 100ml of distilled water add 10g of SDS,

APS

Dissolve 100mg of APS in 1ml of distilled water.

Appendix C

- ***GCF collection and feedback***
- ***Feedback questionnaire for OFCDS***

GCF Sample Collection

Volunteer	Smoking status	Site of collection	Number of samples
1	NON - Female	UPPER RIGHT BUCCAL SULCUS	3
2	NON - Female	" "	"
3	NON - Female	" "	"
4	NON - Female	" "	"
5	NON - Female	" "	"
6	SMOKER - MALE	" "	"
7	NON-SMOKER MALE	" "	"
8	SMOKER - FEMALE	" "	"
9	SMOKER FEMALE	" "	"
10	SMOKER FEMALE	" "	"
11	SMOKER FEMALE	" "	"
12	SMOKER FEMALE	" "	"
13	SMOKER MALE	" "	"
14	SMOKER MALE	" "	"
15	SMOKER MALE	" "	"
16	SMOKER MALE	" "	"
17	NSMOKER MALE	" "	"
18	NON-SMOKER MALE	" "	"
19	NON - SMOKER MALE	" "	"
20	NON - SMOKER MALE	" "	"

GCF Sample Collection

Volunteer	Aesthetics	Ease of Collection	Comfort	Overall experience	
1	10	10	10	10	-GOOD
2	10	10	10	10	-ok
3	10	10	10	10	ok
4	10	10	10	10	ok
5	10	10	10	10	GOOD
6	10	10	10	10	GOOD
7	10	10	10	10	ok
8	10	10	10	10	NO Problem could feel anything
9	10	10	10	10	
10	10	10	10	10	no probs
11	10	10	10	10	ok
12	10	10	10	10	ok
13	10	10	10	10	ok
14	10	10	10	10	ok
15	10	10	10	10	ok
16	10	10	10	10	ok
17	10	10	10	10	ok
18	10	10	10	10	ok
19	10	10	10	10	ok
20	10	10	10	10	ok.

(1) Female, 19
non-Smoker.

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	[redacted]
Smoking Status	[redacted]

Please tick appropriate box:

Dental visits	Regular ✓	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience	
Method 1	[redacted]	3	4	[redacted]3	
Method 2	[redacted]5	4	4	4	- didn't like taste but will do again
Method 3	4	5	3	4	- tastes really bad
Method 4	5	5	6	5	- liked how you could roll it around
Method 5	3	3	4	3	- hard to use
Method 6	7	7	6	6	- volume indicator was good but plastic was a bit hard
Method 7	NULL				

Further Comments (please continue overleaf if required)

Version 3.0

(2) Female, 28
non-smoker.

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	[redacted]
Smoking Status	[redacted]

Please tick appropriate box:

Dental visits	Regular	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience
Method 1	X	10	10	10
Method 2	8	9	9	9
Method 3	9	10	10	10
Method 4	9	10	10	10.
Method 5	6	8	7	7 ← feel pain and hurt abit. difficult to handle.
Method 6	8	7	10	7
Method 7	NULL			

Further Comments (please continue overleaf if required)

Version 3.0

Female, 20
(3) Non-Smoker,

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	[redacted]
Smoking Status	

Please tick appropriate box:

Dental visits	Regular	Fairly regular	Ocassionaly ✓	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience	
Method 1	———	10	10	10	
Method 2	10	10	8	10	The taste is quite bad.
Method 3	7	9	6	8	Very bad taste
Method 4	6	10	9	10	slight after taste
Method 5	6	6	5	6	too big, cotton falls off,
Method 6	10	10	9	10	the collection pad is a bit hard. But overall it's the best
Method 7	NULL				

Further Comments (please continue overleaf if required)

Version 3.0

(4) Female, 21
Non-Smoker.

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	[redacted]
Smoking Status	[redacted]

Please tick appropriate box:

Dental visits	Regular	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience
Method 1	X	6	5	6
Method 2	7	7	7	7
Method 3	5	6	4	5
Method 4	7	8	8	8
Method 5	5	5	5	5
Method 6	8	8	8	8
Method 7	NULL			

Further Comments (please continue overleaf if required)

Method 5 : the two white parts fall in the mouth

Version 3.0

9 Female, 21
Non-Smoker.

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	[redacted]
Smoking Status	[redacted]

Please tick appropriate box:

Dental visits	Regular	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience
Method 1	X	4	4	4
Method 2	6	5	5	5
Method 3	5	7	4	6
Method 4	7	8	7	7
Method 5	5	7	6	7
Method 6	7	7	7	7
Method 7	NULL			

Further Comments (please continue overleaf if required)

Version 3.0

(6) Male, 21, Smoker.

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	Erwan Le Doux
Smoking Status	from time to time

Please tick appropriate box:

Dental visits	Regular	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience
Method 1	X4	5	3	4
Method 2	6	7	5	6
Method 3	5	7	4	5
Method 4	6	7	6	6
Method 5	4	6	5	5
Method 6	7	7	6	7
Method 7	NULL			

Further Comments (please continue overleaf if required)

Version 3.0

(7) Male, 30
Non-smoker.

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	[redacted]
Smoking Status	

Please tick appropriate box:

Dental visits	Regular	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience
Method 1	X	4	4	4
Method 2	6	7	7	7.
Method 3	3	3	3	3.
Method 4	7	8	8	8
Method 5	6	7	7	7.
Method 6	7	7	7	7
Method 7	Not.			

Further Comments (please continue overleaf if required)

Version 3.0

(8) Female, 20
Smoker.

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	[redacted]
Smoking Status	[redacted]

Please tick appropriate box:

Dental visits	Regular ✓	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience
Method 1		5	5	5
Method 2	5	8	~~6~~	7
Method 3	3	6	3	4
Method 4	7	9	9	9
Method 5	7	6	6	6
Method 6	9	8	8	8
Method 7	[illegible]			

Further Comments (please continue overleaf if required)

Version 3.0

(9) Female, 25
Smoker.

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	[redacted]
Smoking Status	

Please tick appropriate box:

Dental visits	Regular	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience	
Method 1		8	8	easy to do	8
Method 2	4	4	6	didn't like it in my mouth.	6
Method 3	6	7	7	OK.	7
Method 4	6	7	5	Bad tasting	5
Method 5	6	7	6	OK around the gums.	6
Method 6	~~6~~ 7	7	7	OK.	7
Method 7	NULL				

Further Comments (please continue overleaf if required)

Version 3.0

(10) Female, 25
Smoker.

[redacted]

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	
Smoking Status	[redacted]

Please tick appropriate box:

Dental visits	Regular	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience
Method 1	—	7	9	8
Method 2	8	5	5	5
Method 3	9	7	7	7
Method 4	6	6	6	6
Method 5	6	7	7	7
Method 6				
Method 7	NULL			

Further Comments (please continue overleaf if required)

Version 3.0

[redacted]

Female, 22
Smoker.

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	[redacted]
Smoking Status	[redacted]

Please tick appropriate box:

Dental visits	Regular ✓	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience
Method 1	-	9	10	9
Method 2	9	9	8	8
Method 3	9	9	9	9
Method 4	7	9	7	7
Method 5	8	8	8	8
Method 6	7	9	7	7
Method 7	NULL			

Further Comments (please continue overleaf if required)

Version 3.0

(12)
[redacted]
Female, 25
Smoker.

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	[redacted]
Smoking Status	[redacted]

Please tick appropriate box:

Dental visits	Regular	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience
Method 1	—	8	8	8
Method 2	9	8	6	7
Method 3	9	8	9	9
Method 4	9	7	7	8
Method 5	8	8	8	8
Method 6	9	9	9	9
Method 7	NULL			

Further Comments (please continue overleaf if required)

Version 3.0

(15) Male, 23
Smoker.

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	[redacted]
Smoking Status	

Please tick appropriate box:

Dental visits	Regular	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience
Method 1	6	4	5	4
Method 2	6	6	6	7
Method 3	6	5	4	4
Method 4	6	6	7	7
Method 5	5	5	4	5
Method 6	6	6	6.5	6
Method 7	NULL			

Further Comments (please continue overleaf if required)

Version 3.0

(19) Male 21, Smoker.

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	[redacted]
Smoking Status	

Please tick appropriate box:

Dental visits	Regular	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience
Method 1	7	5	5	6
Method 2	9	7	6	8
Method 3	9	8	6	9
Method 4	7	9	8	7
Method 5	6	5	7	6
Method 6	9	7	7	8
Method 7	NULL			

Further Comments (please continue overleaf if required)

Version 3.0

Smoker.

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	[redacted]
Smoking Status	

Please tick appropriate box:

Dental visits	Regular	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience
Method 1	4	4 6	5	6
Method 2	7	7	6	7
Method 3	8	8	6	6
Method 4	7	9	8	5
Method 5	7	7	7	6
Method 6	9	7	7	7
Method 7	NULL			

Further Comments (please continue overleaf if required)

Version 3.0

[illegible]
Smoker.

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	[redacted]
Smoking Status	[redacted]

Please tick appropriate box:

Dental visits	Regular	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience
Method 1	4	6	6	6
Method 2	8	7	7	5
Method 3	7	8	7	8
Method 4	7	8	7	8
Method 5	7	6	7	6
Method 6	8	8	8	7
Method 7	NULL			

Further Comments (please continue overleaf if required)

Version 3.0

Non-Smoker

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	[redacted]
Smoking Status	

Please tick appropriate box:

Dental visits	Regular	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience
Method 1	5	7	5	7
Method 2	5	8	7	6
Method 3	7	8	6	6
Method 4	8	7	7	6
Method 5	6	7	6	7
Method 6	7	8	9	5
Method 7	NULL			

Further Comments (please continue overleaf if required)

Version 3.0

non - smoker.

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	[redacted]
Smoking Status	

Please tick appropriate box:

Dental visits	Regular	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience
Method 1	8	4	6	5
Method 2	9	6	8	8
Method 3	8	7	8	8
Method 4	6	7	6	7
Method 5	8	8	7	5
Method 6	7	6	8	7
Method 7	NULL			

Further Comments (please continue overleaf if required)

Version 3.0

(19) Non-Smoker

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	[redacted]
Smoking Status	

Please tick appropriate box:

Dental visits	Regular	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience
Method 1	6	6	7	6
Method 2	7	7	5	7
Method 3	7	9	5	8
Method 4	7	9	8	7
Method 5	8	6	7	6
Method 6	9	7	6	8
Method 7	NULL			

Further Comments (please continue overleaf if required)

(20) male, 27
non-smoker.

UNIVERSITY OF LIVERPOOL

An assessment of different oral swabbing methods in the collection of whole saliva

Feedback Questionnaire

Participant number	[redacted]
Smoking Status	

Please tick appropriate box:

Dental visits	Regular	Fairly regular	Ocassionaly	Hardly	Never

Feedback on swabbing methods (Scale 1-10 1= very poor ; 10= excellent)

	Aesthetics	Ease of collection	Comfort	Overall experience
Method 1	6	6	6	6
Method 2	7	7	4	7
Method 3	7	9	8	6
Method 4	8	8	8	6
Method 5	5	7	6	6
Method 6	8	8	7	7
Method 7	NULL			

Further Comments (please continue overleaf if required)

Version 3.0

All questionnaires beyond this point were done on a scale of 1-5. For purposes of the study all values were doubled to coincide with the 1-10 scale used.

Smoker

Participant

Age 26

Sex Male

Method	Comfort	Ease of Use	Looks	Experience
Saliva	3	5	1	2
Salivette	1	4	3	2
Device	1	1	1	1
Concateno	3	3	5	3
Oracol	4	5	5	4
Orasure	5	5	5	5

Key: rating 1-5 (5= Highest)

Further Comments

Age 30 Smoker

Sex M

Method	Comfort	Ease of Use	Looks	Experience
Saliva	4	5	1	4
Salivette	2	5	3	3
Device	1	3	2	2
Concateno	3	5	3	3
Oracol	4	5	3	4
Orasure	2	5	4	3

Key: rating 1-5 (5= Highest)

Further Comments

Participant NON-SMOKER

Age 21

Sex F.

Method	Comfort	Ease of Use	Looks	Experience
Saliva	2	3		2
Salivette	3	5	3	3
Device	2	2	3	2
Concateno	4	4	4	4
Oracol	4	4	3	4
Orasure	4	4	3	4

Key: rating 1-5 (5= Highest)

Further Comments

Participant [illegible]

Age 30 Smoker

Sex M

Method	Comfort	Ease of Use	Looks	Experience
Saliva	4	5	1	4
Salivette	2	5	3	3
Device	1	3	2	2
Concateno	3	5	3	3
Oracol	4	5	3	4
Orasure	2	5	4	3

Key: rating 1-5 (5= Highest)

Further Comments

Age 24 (SMOKER)

Sex MALE

Method				
	Comfort	Ease of Use	Looks	Experience
Saliva	5	5	2	4
Salivette	2	4	5	3
Device	3	1	2	3
Concateno	3	3	4	4
Oracol	4	4	4	4
Orasure	2	3	3	3

Key: rating 1-5 (5= Highest)

Further Comments

Participant

Age 21

Sex Male

Method	Comfort	Ease of Use	Looks	Experience
Saliva	3	3		4
Salivette	2	3	3	2
Device	1	1	3	2
Concateno	3	4	4	3
Oracol	2	3	3	3
Orasure	3	4	4	3

Key: rating 1-5 (5= Highest)

Further Comments

Participant 4

Age 30 years.

Sex Female – Non Smoker.

Method	Comfort	Ease of Use	Looks	Experience
Saliva				
Salivette	not very Comfort (2)	Easy (5)	ok (4)	(3)
Device	(2)	(2)	(2)	(2)
Concateno	(4)	(4)	(4)	(4)
Oracol	(3)	(4)	(4)	(4)
Orasure	(3)	(4)	(4)	(4)

Key: rating 1-5 (5= Highest)

Further Comments

- orasure is tasty.
- Salivette is not very Comfort.

Participant

Age 30

Sex Female - Non smoker

Method	Comfort	Ease of Use	Looks	Experience	
Saliva	~~4~~	5	3	3	Definately need water. Takes quite a while to get 5ml
Salivette	5	5	5	~~4~~	like chewing cotton wool, but done quickly.
Device	~~3~~	3	3	3	pads do not stay in place well. Not easy to get into position. Could do with a cover to protect sample
Concateno	5	5	5	5	like the indicator to tell you enough has been collected. Very sterile.
Oracol	5	5	4	5	
Orasure	3	~~4~~	4	3	Good instructions on packaging

Key: rating 1-5 (5= Highest)

Further Comments

Participant

Age 22

Sex MALE

Method	Comfort	Ease of Use	Looks	Experience	
Saliva	5	5	5	3	
Salivette	3	4	4	3	
Device	3	3	4	4	Comparatively easy method ☺
Concateno	4	4	4	4	No problem experienced during experiment
Oracol	3	3	4	2	
Orasure	3	2	3	2	Because of taste experiment was not so good.

Key: rating 1-5 (5= Highest)

Further Comments

NON - SMOKER

Participant

Age 26 Non-Smoker

Sex M

	Method	Comfort	Ease of Use	Looks	Experience
a	Saliva	2	3	1	2
b	Salivette	5	5	3	5
c	Device	2	3	4	3
d	Concateno	3	3	5	3
e	Oracol	2	3	3	3
f	Orasure	1	2	3	2

Key: rating 1-5 (5= Highest)

Further Comments

(a) General feelings — not particularly pleasant. Not easy to aim in tube.

(b) easy to use. Produced much more saliva than (a)

(c) Saliva pools in mouth. Not v. comfortable. also sticks to gum a bit.

(d) Took a long time to turn blue. Not comfortable on gums.

(e) Abrasive on tongue. leaving it in mouth after is annoying.

(f) Saltiness strength pleasant at first. Riddly to open package. Saltiness unpleasant when had to leave in mouth for a minute → bitter like

Hard to put in tube

Aftertaste of salt not nice.

Participant

Age 22

Sex MALE

Method	Comfort	Ease of Use	Looks	Experience
Saliva	5	5	5	3
Salivette	3	4	4	3
Device	3	3	4	4
Concateno	4	4	4	4
Oracol	3	3	4	2
Orasure	3	2	3	2

Key: rating 1-5 (5= Highest)

Further Comments

NON-SMOKER

Participant

Age 30 Years.

Sex Female – Non Smoker.

Method	Comfort	Ease of Use	Looks	Experience
Saliva				
Salivette	not very comfort (3)	Easy (5)	ok (4)	(3)
Device	(2)	(2)	(2)	(2)
Concateno	(4)	(4)	(4)	(4)
Oracol	(3)	(4)	(4)	(4)
Orasure	(3)	(4)	(4)	(4)

Key: rating 1-5 (5= Highest)

Further Comments

Participant

Age 26

Sex Male

Method				
	Comfort	Ease of Use	Looks	Experience
Saliva	3	5	1	2
Salivette	1	4	3	2
Device	1	1	1	1
Concateno	3	3	5	3
Oracol	4	5	5	4
Orasure	5	5	5	5

Key: rating 1-5 (5= Highest)

Further Comments

Participant

Age 21

Sex Female

Smoker

Method	Comfort	Ease of Use	Looks	Experience
Saliva	2	3		3
Salivette	1	3	2	2
Device	1	1	2	1
Concateno	3	4	3	3
Oracol	5	5	4	5
Orasure	4	4	4	4

Key: rating 1-5 (5= Highest)

Further Comments

Participant

Age 27 SMOKER

Sex FEMALE

Method	Comfort	Ease of Use	Looks	Experience
Saliva	5	5	N/A	5
Salivette	3	4	3	3
Device	1	1	1	1
Concateno	3	4	4	4
Oracol	3	5	3	3
Orasure	1	3	2	2

Key: rating 1-5 (5= Highest)

Further Comments